APPENDIX A

The claims 39-41, 43-48, 54-55, 57-58, 60-69 and 71-95 involved in this appeal are as follows:

39.   A method for producing a functional antibody comprising a heavy chain and a light chain, which comprises the steps of:

(a)   transfecting a non-antibody producing mammalian cell with a first DNA sequence coding for a first chain of the antibody;

(b)   transfecting the cell with a second DNA sequence, said second DNA sequence coding for a second chain of the antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain, and

(c)   maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA sequences and the resultant chains are intracellularly assembled together to form the antibody which is then secreted in a form capable of specifically binding to antigen.

40.   A method as recited in claim 39 wherein the cell is transfected via protoplast fusion.

41.   A method as recited in claim 39 wherein the cell is transfected via calcium phosphate precipitation.

43. A method as recited in claim 78 wherein the cell is a myeloma cell.

44. A method as recited in claim 43 wherein the cell is a murine myeloma cell.

45. A method as recited in claim 39 wherein the cell does not endogenously produce any immunoglobulin chains.

46. A method as recited in claim 45 wherein the cell is a murine P_3 cell.

47. A method as recited in claim 39 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

48. A method as recited in claim 47 wherein the cell is a murine J558L cell.

54. A method for producing a functional antibody comprising a heavy chain and a light chain, which comprises the steps of:

(a) transfecting a non-antibody producing mammalian cell with a plasmid comprising a first DNA sequence coding for a first chain of the antibody and a second DNA sequence coding for a second chain of the antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(b)   maintaining the cell in a nutrient medium so that the cell expresses said first DNA sequence and said second DNA sequence and the resultant chains are intracellularly assembled together to form the antibody which is then secreted in a form capable of specifically binding to antigen.

55.   A method as recited in claim 39 wherein the antibody is a chimeric antibody comprising a variable region substantially the same as that found in a first mammalian source and comprising a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

57.   A method as recited in claim 54 wherein the cell is transfected via protoplast fusion.

58.   A method as recited in claim 54 wherein the cell is transfected via calcium phosphate precipitation.

60.   A method as recited in claim 84 wherein the cell is a myeloma cell.

61.   A method as recited in claim 60 wherein the cell is a murine myeloma cell.

62.   A method as recited in claim 54 wherein the cell does not endogenously produce any immunoglobulin chains.

63.   A method as recited in claim 62 wherein the cell is a murine P_3 cell.

64.   A method as recited in claim 54 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

65.   A method as recited in claim 64 wherein the cell is a murine J558L cell.

66.   A method as recited in claim 54 wherein the antibody is a chimeric antibody comprising a variable region substantially the same as that found in a first mammalian source and comprising a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

67.   A method for producing a functional antibody comprising a heavy chain and a light chain which comprises the steps of:

(a)   maintaining in a nutrient medium a non-antibody producing mammalian cell, said cell having been transfected with a first DNA sequence coding for a first chain of the antibody and a second DNA sequence coding for a second chain of the

4

antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain;

(b)   expressing from said cell the heavy chain and the light chain functionally assembled together to form said antibody which is then secreted in a form capable of binding antigen; and

(c)   recovering said antibody.

68.   A method as recited in claim 67 wherein the cell is transfected via protoplast fusion.

69.   A method as recited in claim 67 wherein the cell is transfected via calcium phosphate precipitation.

71.   A method as recited in claim 90 wherein the cell is a myeloma cell.

72.   A method as recited in claim 90 wherein the cell is a murine myeloma cell.

73.   A method as recited in claim 67 wherein the cell does not endogenously produce any immunoglobulin chains.

74.   A method as recited in claim 73 wherein the cell is a murine P_3 cell.

75.   A method as recited in claim 67 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

76.   A method as recited in claim 75 wherein the cell is a murine J558L cell.

77.   A method as recited in claim 67 wherein the antibody is a chimeric antibody comprising a variable region substantially the same as that found in a first mammalian source and comprising a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

78.   A method for producing a functional antibody comprising a heavy chain and a light chain, which comprises the steps of:

(a)   transfecting a non-antibody producing lymphoid cell with a first DNA sequence coding for a first chain of the antibody;

(b)   transfecting the cell with a second DNA sequence, said second DNA sequence coding for a second chain of the antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(c)   maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA sequences and the resultant chains are intracellularly assembled

together to form the antibody which is then secreted in a form capable of specifically binding to antigen.

79.   A method as recited in claim 78 wherein the cell is transfected via protoplast fusion.

80.   A method as recited in claim 78 wherein the cell is transfected via calcium phosphate precipitation.

81.   A method as recited in claim 78 wherein the cell does not endogenously produce any immunoglobulin chains.

82.   A method as recited in claim 78 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

83.   A method as recited in claim 78 wherein the antibody is a chimeric antibody having a variable region substantially the same as that found in a first mammalian source and having a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

84.   A method for producing a functional antibody comprising a heavy chain and a light chain, which comprises the steps of:

(a)   transfecting a non-antibody producing lymphoid cell with a plasmid comprising a first DNA sequence coding for a first chain of the antibody and a second DNA sequence coding for a second chain of the antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(b)   maintaining the cell in a nutrient medium so that the cell expresses said first DNA sequence and said second DNA sequence and the resultant chains are intracellularly assembled together to form the antibody which is then secreted in a form capable of specifically binding to antigen.

85.   A method as recited in claim 84 wherein the cell is transfected via protoplast fusion.

86.   A method as recited in claim 84 wherein the cell is transfected via calcium phosphate precipitation.

87.   A method as recited in claim 84 wherein the cell does not endogenously produce any immunoglobulin chains.

88.   A method as recited in claim 84 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

89. A method as recited in claim 84 wherein the antibody is a chimeric antibody comprising a variable region substantially the same as that found in a first mammalian source and comprising a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

90. A method for producing a functional antibody comprising a heavy chain and a light chain which comprises the steps of:

(a) maintaining in a nutrient medium a non-antibody producing lymphoid cell, said cell having been transfected with a first DNA sequence coding for a first chain of the antibody and a second DNA sequence coding for a second chain of the antibody, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain;

(b) expressing from said cell the heavy chain and the light chain functionally assembled together to form said antibody which is then secreted in a form capable of binding antigen; and

(c) recovering said antibody.

91. A method as recited in claim 90 wherein the cell is transfected via protoplast fusion.

92. A method as recited in claim 90 wherein the cell is transfected via calcium phosphate precipitation.

93.   A method as recited in claim 90 wherein the cell does not endogenously produce any immunoglobulin chains.

94.   A method as recited in claim 90 wherein the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain but not both.

95.   A method as recited in claim 90 wherein the antibody is a chimeric antibody comprising a variable region substantially the same as that found in a first mammalian source and having a constant region substantially the same as that found in a second mammalian source, said second mammalian source being from a mammalian species other than that of the first mammalian source.

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO

EXAMINER

RECEIVED

ART UNIT	PAPER NUMBER
	41

JUN 16 1995

FISH & NEAVE — PATENT DEPT. DATE MAILED:
REFERRED TO_____
NOTED BY_____

This is a communication from the examiner in charge of your application
COMMISSIONER OF PATENTS AND TRADEMARKS

☑ This application has been examined ☒ Responsive to communication filed on 3/16/95 ☒ This action is made final

A shortened statutory period for response to this action is set to expire three month(s). _____ days from the date of this letter
Failure to respond within the period for response will cause the application to become abandoned 35 U.S C 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892   2. ☐ Notice of Draftsman's Patent Drawing Review, PTO-948
3. ☐ Notice of Art Cited by Applicant, PTO-1449   4. ☐ Notice of Informal Patent Application, PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474. 6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 39-41, 43-48, 54-55, 57-58, 66-69 and 71-95 are pending in the application

Of the above, claims _____ are withdrawn from consideration

2. ☐ Claims _____ have been cancelled

3. ☐ Claims _____ are allowed

4. ☐ Claims _____ are rejected

5. ☐ Claims _____ are objected to

6. ☐ Claims _____ are subject to restriction or election requirement

7. ☐ This application has been filed with informal drawings under 37 C F R 1 85 which are acceptable for examination purposes

8. ☐ Formal drawings are required in response to this Office action

9. ☐ The corrected or substitute drawings have been received on _____ Under 37 C F R 1 84 these drawings
    are ☐ acceptable; ☐ not acceptable (see explanation or Notice of Draftsman's Patent Drawing Review, PTO-948)

10. ☐ The proposed additional or substitute sheet(s) of drawings filed on _____ has (have) been ☐ approved by the
    examiner, ☐ disapproved by the examiner (see explanation)

11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation)

12. ☐ Acknowledgement is made of the claim for priority under 35 U S C 119 The certified copy has ☐ been received ☐ not been received
    ☐ been filed in parent application, serial no _____ ; filed on _____

13. ☐ Since this application apppears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in
    accordance with the practice under Ex parte Quayle, 1935 C.D 11, 453 O G 213

14. ☐ Other

DOCKETED FOR _____

EXAMINER'S ACTION

III. DETAILED ACTION

15.   The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

16.   Claims 39-41, 43-48, 54-55, 57-58, 60-69, and 71-95 are rejected under 35 U.S.C. § 103 as being unpatentable over Cabilly (L,R, or 2A) in view of Gillies (Cell 1983).

Rejections are maintained for reasons of record, stated in papers 5, 7, and 10, mailed November 29, 1988, May 24, 1989, and September 25, 1990.

In the response filed 3/16/95, applicants argue that the motivation to combine Gillies and Cabilly does not exist. Cabilly is criticized for failing to actually teach double transfection. Applicants urge that mere recitation of mammalian cells as a host is not sufficient to motivate one of ordinary skill in the art to actually combine Cabilly with Gillies. Gillies is traversed as failing to produce a functional antibody because the transfected heavy chain is of different specificity than the Traversal continues with the argument that the exepectation of success is similarly lacking.

These arguments have been considered but are not deemed persuasive. The argument concerning Cabilly is not considered persuasive because of the aforementioned specific teaching of producing antibodies in mammalian cells. See Cabilly (L), pages 18 and 19, for example. While applicants are correct in characterizing Cabilly's disclosure as non-enabling for myeloma cell production, Cabilly is only used to teach double transfection. Gillies shows the production of antibodies in myeloma cells. Morover, Gillies teaches the production of proteins in yields approaching wild type. Therefore, such yields are considered approximating 100% in comparison to applicant's 32%. Accordingly, applicant's argued unexpected yields are not considered as such. As far as applicant's argument regarding the fact that Gillie's yield is of unassembled protein, such is simply not supported by Gillies. Applicants have not pointed out the statement in Gillies where the routineer would learn that Gillies' protein is not assembled. Absent such a clear disclosure to the contrary, it is more reasonable to assume that Gillies' disclosed yield is functional. Otherwise, Gillies would not be producing the protein. The protein/antibody is of no use when it is not functional.

Applicant's new arguments concerning the alleged failure of Gillies to produce functional antibodies(the exogenous heavy chain is of different specificity than the endogenous light chain, hence the two chains are not complementary) is noted. However, no evidence exists to support applicant's allegation in the 3/16/95 response of nonfunctionality. Moreover, review of the

Serial No.: 08/266,154
Art Unit: 1806

Gillies reference does not support applicant's conclusion regarding nonfunctionality. As applicants have stated, Gillies is primarily concerned with the enhancer element, not the actual production of the antibody. Therefore, the reference is basically silent on the issue of heavy chain specificity. Therefore, no evidence exists on the record to support applicant's assertion of nonfunctionality. Further, assuming *arguendo*, that the heavy chain in Gillies is of a separate specificity than the endogenous light chain, one of ordinary skill in the art would merely have to substitute a complementary heavy chain for that set forth in Gillies. The routineer would be motivated to do so because transfection with a complementary would be necessary to yield a binding antibody. Thus Gillies is applied for what it reasonably discloses. That disclosure is the ability of myeloma cells to express, assemble, and secrete exogenous antibody chain. Accordingly, the rejection is maintained for reasons of record.

17. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

18. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Nisbet whose telephone number is (703) 308-4204 from 9:00 am to 5:00 pm weekdays with the exception of alternating Mondays. If the examiner cannot be reached, the supervisor, Margaret Moskowitz Parr, may be contacted at phone number (703)308-2454.

The number for facsimile submission of papers has changed. The new fax number for Art Unit 1806 is (703) 305-7401. Please provide the serial number, application title, examiner's name, and art unit on the fax cover sheet to expedite clerical processing. In addition, all cover sheets should be marked **DRAFT** or **OFFICIAL** as appropriate.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

MARGARET PARR
SUPERVISOR PATENT EXAMINER
GROUP 1800

EXHIBIT C

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
07/090,669	08/28/87	MORRISON	24302/STAN-62-1

LEYDIG, VOIT & MAYER
350 CAMBRIDGE AVENUE
SUITE 200
PALO ALTO, CA 94306

RECEIVED.

DEC 0 8 1988

LEYDIG, VOIT & MAYER
PALO ALTO

EXAMINER
MARKS,M

ART UNIT	PAPER NUMBER
185	5

DATE MAILED: 11/29/88

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

This application has been examined ☐ Responsive to communication filed on _____ ☐ This action is made final.

Shortened statutory period for response to this action is set to expire __3__ month(s), _____ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.
2. ☒ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☒ PTOL-413

SUMMARY OF ACTION

☐ Claims _____1-30_____ are pending in the application.

Of the above, claims _____1-13_____ are withdrawn from consideration.

☐ Claims _____ have been cancelled.

☐ Claims _____ are allowed.

☐ Claims _____14-38_____ are rejected.

☐ Claims _____ are objected to.

☐ Claims _____1-38_____ are subject to restriction or election requirement.

☐ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.

☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.

☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).

☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.

☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

☐ Other

RESPONSE DUE 12-28-89

EXAMINER'S ACTION   12/4/88   PMK

PTOL-326 (Rev. 7-82)

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-13, drawn to chimeric receptors, classified in Class 530, subclass 387.

II. Claims 14-38, drawn to the DNA, mammalian cell line and methods of preparing the receptor, classified in Class 435, subclass 172.3+.

The inventions are distinct, each from the other because of the following reasons:

Inventions [Group II] and [Group I] are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make other and materially different products or (2) that the product as claimed can be made by another and materially different process (MPEP 806.05(f)). In the instant case the product as claimed can be made by another and materially different process such as protein synthesis.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as shown by their different classification, restriction for examination purposes as indicated is proper.

During a telephone conversation with Bertan Rawland on October 4,
1988 a provisional election was made with traverse to prosecute
the invention of Group II, claims 14-38. Affirmation of this
election must be made by applicant in responding to this office
action. Claims 1-13 are withdrawn from further consideration by
the examiner, 37 CFR 1.142(b), as being drawn to a non-elected
invention.

Claims 14-38 are rejected under 35 U.S.C. 112, first
paragraph, as the disclosure is enabling only for claims limited
to DNA constructs for expression of a chimeric polypeptide which
is a subunit of an immunoglobulin molecule. See MPEP 706.03(n)
and 706.03(z).

Subunits of other multi-unit receptors were not enabled (eg.
IgE receptors, IL-1 and IL-2 receptors, fibronectin receptors and
other integrins). It would require undo experimentation to
determine the variable and constant region amino acid and genomic
sequence homolgies of these receptors for use in cross- species
chimeric constructions.

Claims 14, 28, 33, and 36 are rejected under 35 U.S.C. 112,
second paragraph, as being indefinite for failing to
particularly point out and distinctly claim the subject matter
which applicant regards as the invention.

Claims 14, 28 and 36 are indefinite by claiming constructs

for a multi-unit receptor, cells, and a method for expressing a multi-unit receptor. Not all multi-unit receptors are taught in the instant invention. Is immunoglobulin intended rather than the genus multi-unit receptor? If not, which multi-unit receptor is intended (eg. an integrin, an IL-1 or IL-2 receptor, or an IgE receptor)? Binding activities are associated with numerous multi-chain polypeptides (ie. receptor). Please clarify what is intended by the multi-unit receptor.

Claims 28 and 33 are confusing and obscuring in the recitation " a mammalian cell having first and second DNA constructs for expression of different first and second subunits... each of said first and second constructs comprising...". Since the first and second constructs are comprised of the same components, they may be one in the same. Wording such as "a mammalian cell having different first and second DNA constructs..." would clarify this claim.

Claims 25-27 are rejected under 35 U.S.C. 112, fourth paragraph, as being of improper dependent form for failing to further limit the subject matter of a previous claim.

Claim 25 refers to a promoter in claims 15 or 16 while no promoter is specifically mentioned in either claim 15 or 16. Claims 26 and 27 fail to further define claims 14-16 by including an additional element (ie. a replication system).

The following is a quotation of the appropriate paragraphs of 35
U.S.C. 102 that form the basis for the rejections under this
section made in this Office Action:

A person shall be entitled to a patent unless—

(a) the invention was known or used by others in this
country, or patented or described in a printed publication in
this or a foreign country, before the invention thereof by the
applicant for a patent.

A person shall be entitled to a patent unless

(b) the invention was patented or described in a
printed publication in this or a foreign country or in public use
or on sale in this country, more than one year prior to the date
of application for patent in the United States.

Claims 14-34 and 36 are rejected under 35 U.S.C. 102(b) as being

clearly anticipated by Cabilly (L).

Cabilly teaches various DNA constructs for the expression of

chimeric immunoglobulin heavy chains, chimeric immunoglobulin

light chains, chimeric immunoglobulins, and chimeric

immunoglobulin fragments (ie. chimeric subunits of a multiunit

receptor). Cabilly teaches various expression vectors for use in

either prokaryotic or eukaryotic host cells (See page 15 lines 6-

29). Cabilly also teaches the use of eukaryotic host cells,

including various mammalian cell types (page 18 line 6-34 and

page 19 lines 1-8). Further, Cabilly teaches the use of the

regulatory elements required for expression in vertebrate cells

(page 18, lines 11-14). Finally Cabilly teaches the coexpression

of both the heavy and light chains of immunoglobulin in the same

host (page 23 line 29-30). Thus, all aspects of the instant

invention, except for expression specifically in murine myeloma

cells, was taught by Cabilly.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office Action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

> Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Claims 35, 37-38 are rejected under 35 U.S.C. 103 as being unpatentable over Cabilly(L).

Cabilly teaches the use of eukaryotic host cells , including vertebrate cell hosts (pages 18 and 19). In the absence of unexpected results, it would be obvious to use myeloma cells as a type of mammalian cell as taught by Cabilly. It would be particularly obvious to use myeloma cells to express a DNA construct whose major regulatory and coding sequences are endogenous to said myeloma cell, since the endogenous host would contain the both 1) the trans-acting factors necessary for activating immunoglobulin cis elements and 2) the cytoplasmic organelles and enzymes required for co- and post-translational

processing of immunoglobulin chains.

Claims 14-38 are rejected under 35 U.S.C. 103 as being unpatentable over Gillies (S) as applied to claims 35, 37 and 38 above, and further in view of Cabilly (L).

Cabilly is applied as described above. It would be obvious to use mammalian cells, particularly myeloma cells, to express a chimeric polypeptide whose components are endogenous to said myeloma cells. Gillies teaches the use of mammalian cells (ie. myeloma cells) as well as the method for expressing immunoglobulin genes in myeloma cells. In the absence of unexpected results, it would be obvious to one of ordinary skill in the art to utilize the vector and cells of Gillies to express the chimeric constructs of Cabilly in order to produce the chimeric receptor (ie. antibody or immunoglobulin fragment) of Cabilly.

Please note the following references as being pertinent to the state of the art: Murphy (M), Seno(T), and Dolby (U).

Any inquiry concerning this communication should be directed to Examiner Michelle Marks, Ph.D. at telephone number 703-557-0664.

11.2.88 msm

FORM PTO-892 (REV. 3-78)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO. 07/090,669	GROUP ART UNIT 185	ATTACHMENT TO PAPER NUMBER
NOTICE OF REFERENCES CITED		APPLICANT(S) MORRISON		

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PP. SPEC.
	L	0125023	14 Nov 84	EP	CABILLY	C12N	15/00		
	M	9303971	24 Nov 93	WO	MURPHY	A6K	37/00		
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R	Cabilly, S. et al 1984 (June) PNAS, USA 91: 3273-3277 Generation of antibody activity from immunoglobulin polypeptide chains produced
S	Gillies SD et al 1983 Cell 33: in Escherichia coli 717-728
T	Sood et al 1983 Nucleic Acid Research 11(3): 719-726
U	D H... et al 1980 PNAS 77(10) 6027-6031 Oct/Aug 1980

EXAMINER MARKS	DATE 11/1/88	

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.05 (a).)

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER

ART UNIT	PAPER NUMBER

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) _Jarnetta Shaefer (Examiner)_ (3) _____

(2) _Bertran Rowland (attorney)_ (4) _____

Date of interview ___10-4-8_

Type: ☒ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☒ No. If yes, brief description: _____

Agreement ☒ was reached with respect to some or all of the claims in question. ☐ was not reached.

Claims discussed: _all_

Identification of prior art discussed: _none_

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: _Mr Rowland_
provisionally elects with traverse to prosecute the
Claims of Group II (14-38).
Also the status of the parent file (644473) was discussed
relative to the suspension and I was pointed out that the case
in which it will probably go into interference with, has been altered

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1–7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☒ It is not necessary for applicant to provide a separate record of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

_____
Examiner's Signature

PTOL-413 (REV. 1-84)

ATTACHMENT TO
PAPER NUMBER

S.N. 090669

GROUP

NOTICE OF PATENT DRAWINGS OBJECTION

Drawing Corrections and/or new drawings may only be
submitted in the manner set forth in the attached letter,
"Information on How to Effect Drawing Changes" PTO-1474.

A. ☑ The drawings, filed on ___8/28/87___, are objected to as informal for reason(s)
checked below:

1. ☐ Lines Pale.

2. ☐ Paper Poor.

3. ☐ Numerals Poor.

4. ☐ Lines Rough and Blurred.

5. ☐ Shade Lines Required.

6. ☑ Figures Must be Numbered. *Separately*
   Fig 1

7. ☐ Heading Space Required.

8. ☐ Figures Must Not be Connected.

9. ☐ Criss-Cross Hatching Objectionable.

10. ☐ Double-Line Hatching Objectionable.

11. ☐ Parts in Section Must Be Hatched.

12. ☐ Solid Black Objectionable.

13. ☐ Figure Legends Placed Incorrectly.

14. ☐ Mounted Photographs.

15. ☐ Extraneous Matter Objectionable.
    [37 CFR 1.84 (1)]

16. ☐ Paper Undersized; either 8½" x 14",
    or 21.0 cm. x 29.7 cm. required.

17. ☐ Proper A4 Margins Required:
    ☐ TOP 2.5 cm.   ☐ RIGHT 1.5 cm.
    ☐ LEFT 2.5 cm.   ☐ BOTTOM 1.0 cm.

18. ☑ Other:
    - Characters must be at
    least ⅛" taller

B. ☑ The drawings, submitted on ___8/28/87___, are so informal they cannot be
corrected. New drawings are required. Submission of the new drawings MUST be
made in accordance with the attached letter.

EXHIBIT D

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO
	08/26/87	MORRISON	24302/STAN-62

LEYDIG, VOIT & MAYER
.50 CAMBRIDGE AVENUE
SUITE .III
PALO ALTO, CA 94306

RECEIVED

MAY 26 1989

L, VOIT & MAY
MR ALT

EXAMINER	
ART UNIT	PAPER NUMBER
	7
DATE MAILED	

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☒ This application has been examined   ☒ Responsive to communication filed on 3/26/89   ☐ This action is made final.

A shortened statutory period for response to this action is set to expire __3__ month(s), _____ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned.   35 U.S.C. 133

Part I   THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.   2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449   4. ☐ Notice of informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474   6. ☐ _____

Part II   SUMMARY OF ACTION

1. ☒ Claims _____14-38_____ are pending in the application.

    Of the above, claims _____ are withdrawn from consideration.

2. ☒ Claims _____1-13_____ have been cancelled.

3. ☐ Claims _____ are allowed.

4. ☒ Claims _____14-38_____ are rejected.

5. ☐ Claims _____ are objected to.

6. ☐ Claims _____ are subject to restriction or election requirement.

7. ☐ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.

8. ☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.

9. ☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).

10. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

12. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____ ; filed on _____

13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

14. ☐ Other

8-24-89
11-24-89

PTOL-326 (Rev. 7 - 82)   EXAMINER'S ACTION

5-28-89 PM
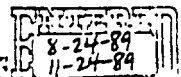
Examiner acknowledges applicants' election of Group II, claims 14-38 without traverse in Paper No. 6.

Claims 14-38 are rejected under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited to DNA constructs for expression of a chimeric polypeptide which is a subunit of an immunoglobulin molecule. See MPEP 706.03(n) and 706.03(z). This rejection is maintained for essentially the same reasons set forth in the previous office action.

Subunits of other multi-unit receptors which contain a variable region and constant region were not enabled (eg. T-cell receptors and Major Histocompatibility Complex antigens). Applicants' response of Paper No. 6 states that T cell receptors and Major Histocompatibility Antigens (MHC) are of the same supergene family as that of immunoglobulins, containing variable and constant regions, and are therefore enabled by the scope of their disclosure. Examiner disagrees with applicants argument that T cell receptors and MHC antigens were enabled in their disclosure because they contain variable and constant regions. The instant specification cites the receptors of interest "include B cell and T cell receptors, more particularly, Immunoglobulins (page 31 lines 23-25) yet does not provide a means for isolating the DNA encoding B and T cell receptors. Not all B and T cell receptors have variable and constant regions (eg CD2, CD7, CD20 and Fc). Cloning and sequencing of MHC antigens

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was not disclosed or enabled in the instant specification. There are three major reasons why T cell receptors and MHC antigens are not considered within the scope of this invention. First, the instant specification does not disclose that these "multi-chain" receptors contain variable and constant regions and fails to describe how the DNA encoding such molecules would be isolated. It is well known to the immunogenetics art that isolating these latter proteins and their DNA was difficult. Second, not all T cell receptors have constant and variable regions. Only T cell receptors specific for antigens are composed of variable and constant regions, of which the variable region proteins were known to be homologous to immunoglobulins at the time of filing this application; but, again, this was not disclosed in the instant specification. However, at the time the instant specification was filed, it was not known that MHC antigens contained variable and constant regions. Third, it would require undo experimentation to isolate variable and constant region genomic sequence of these receptors for use in cross- species chimeric constructions.

Claims 14, 28, 33, and 36 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 14, 28 and 36 are indefinite by claiming constructs

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for a multi-unit receptor, cells, and a method for expressing a
multi-unit receptor. Not all multi-unit receptors are taught in
the instant invention. Is immunoglobulin intended rather than
the genus multi-unit receptor? If DNA constructs encoding the
supergene family of immunoglobulins, T-cell receptors and MHC
antigens was intended, it should be named as such. The instant
specification does not enable isolation of the DNA for both the
constant and variable region genes encoding T cell receptors or
MHC antigens.

The following is a quotation of the appropriate paragraphs
of 35 U.S.C. 102 that form the basis for the rejections under
this section made in this Office Action:

A person shall be entitled to a patent unless-

(b) the invention was patented or described in a printed
publication in this or a foreign country or in public use or on
sale in this country, more than one year prior to the date of
application for patent in the United States.

Claims 14-34 and 36 are rejected under 35 U.S.C. 102(b) or 102
(e) as being clearly anticipated by Cabilly (L) or Cabilly (R)
or Cabilly (2A).

This rejection is a new rejection which is essentially the
same rejection set forth in the previous office action in the
rejection of claims 14-34 and 36 over Cabilly (L).

Each of the Cabilly references cited above teaches various
DNA constructs for the expression of chimeric immunoglobulin
heavy chains, chimeric immunoglobulin light chains, chimeric
immunoglobulins, and chimeric immunoglobulin fragments (ie.

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chimeric subunits of a multiunit receptor). Cabilly teaches various expression vectors for use in either prokaryotic or eukaryotic host cells (See page 15 lines 6-29). Cabilly also teaches the use of eukaryotic host cells, including various mammalian cell types (page 18 line 6-34 and page 19 lines 1-8). Further, Cabilly teaches the use of the regulatory elements required for expression in vertebrate cells (page 18, lines 11-14). Finally Cabilly teaches the coexpression of both the heavy and light chains of immunoglobulin in the same host (page 23 line 29-30). Thus, all aspects of the instant invention, except for expression specifically in murine myeloma cells, was taught by Cabilly.

Note Cabilly (2A) in teaching the production of an immunoglobulin molecule. Note column 24 lines 60-66 and Figure 8C showing that cells double transformed with the construct for each immunoglobulin chain express both heavy and light chain proteins. Column 25 lines 45-68 and column 26 describe the reconstitution of antibody from recombinant heavy and light chains. The table in column 27 shows that the heavy and light chains were reconstituted to form functional immunoglobulins which specifically recognize antigen. 0.76% and 0.33% of the recombinant antibody chains were reconstituted as compared to 0.4% of the hybridoma produced antibody chains. Thus, Cabilly produced a functional chimeric antibody composed of recombinant

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heavy and light chains: Further, Cabilly (R) teaches the

motivation to express pre-light chains and pre-heavy chains in

mammalian cells (page 3277 left paragraph) so that the

immunoglobulin chains will be secreted and assembled in vivo.

The following is a quotation of 35 U.S.C. 103 which forms the
basis for all obviousness rejections set forth in this Office
Action:

> A patent may not be obtained though the invention
> is not identically disclosed or described as set
> forth in section 102 of this title, if the dif-
> ferences between the subject matter sought to be
> patented and the prior art are such that the sub-
> ject matter as a whole would have been obvious at
> the time the invention was made to a person having
> ordinary skill in the art to which said subject
> matter pertains. Patentability shall not be nega-
> tived by the manner in which the invention was
> made.
>
> Subject matter developed by another person, which
> qualifies as prior art only under subsection (f)
> and (g) of section 102 of this title, shall not
> preclude patentability under this section where the
> subject matter and the claimed invention were, at
> the time the invention was made, owned by the same
> person or subject to an obligation of assignment to
> the same person.

Claims 35 and 37-38 are rejected under 35 U.S.C. 103 as being

unpatentable over Cabilly (L) or Cabilly (R) or Cabilly (2A)

in view of Gillies (S).

Cabilly teaches the use of eukaryotic host cells ,

including vertebrate cell hosts (eg L, pages 18 and 19). In the

absence of unexpected results, it would be obvious to use myeloma

cells as a type of mammalian cell as taught by Cabilly. It would

be particularly obvious to use myeloma cells to express a DNA

construct whose major regulatory and coding sequences are endogenous to said myeloma cell, since the endogenous host would contain the both 1) the trans-acting factors necessary for activating immunoglobulin cis elements and 2) the cytoplasmic organelles and enzymes required for co- and post-translational processing of immunoglobulin chains. Further, Cabilly (R) teaches the motivation to express pre-light chains and pre-heavy chains in mammalian cells (page 3277 left paragraph) so that the immunoglobulin chains will be secreted and assembled in vivo. Thus, one of ordinary sill in the art would be motivated to use mammalian cells to secrete immunoglobulin chains which are assembled in vivo. In the absence of unexpected results, one of ordinary skill in the art would be further motivated to use myeloma cells because of their properties discussed above which would be advantageous for expressing immunoglobulins.

Gillies teaches the use of mammalian cells, particularly myeloma cells, as well as the method for expressing immunoglobulin genes in myeloma cells. Applicants note on Paper No. 6 , page 6 that the two of the coinventors of the Gillies patent are co-inventors of the subject application. Therefore, examiner asserts that applicant would be motivated to use these known cells for their known properties. In the absence of unexpected results, it would be obvious to one of ordinary skill in the art to utilize the vector and cells of Gillies to express

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the chimeric constructs of Cabilly in order to produce the chimeric receptor (ie. antibody or immunoglobulin fragment) of Cabilly.

Claims 28-35 are, rejected under 35 U.S.C. 103 as being unpatentable over Boss (2B) in view of Gillies (S).

Boss teaches the method of producing antibodies comprising transforming a host cell with DNA sequence encoding each of a heavy and light chain of an immunoglobulin. Gillies is applied as above in teaching the use of myeloma cells for expressing heterologous products and the particular advantages of myeloma cells in expressing immunoglobulin genes. ONe of ordinary skill in the art would be motivated to use the best host cell system for expressing a desired product. It would be particularly obvious to use the myeloma host cells of Gillies to express a DNA construct whose major regulatory and coding sequences are endogenous to said myeloma cell, since the endogenous host would contain the both 1) the trans-acting factors necessary for activating immunoglobulin cis elements and 2) the cytoplasmic organelles and enzymes required for co- and post-translational processing of immunoglobulin chains. In the absence of unexpected results, it would be obvious to one of ordinary skill in the art to use myeloma cells as host for expressing DNA constructs containing the transcriptional and translational regulatory elements which this cell recognizes specifically.

8

Serial No. 07/090,669
Art Unit 185

Any inquiry concerning this communication should be directed to Examiner Michelle Marks, Ph.D. at telephone number 703-557-0664.

THOMAS G. WISEMAN
SUPERVISORY PATENT EXAMINER
ART UNIT 185

FORM PTO 892 (REV 3-78)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO 0591149	GROUP ART UNIT 185	ATTACHMENT TO PAPER NUMBER 7

NOTICE OF REFERENCES CITED

APPLICANT(S) Morrison et al

U.S. PATENT DOCUMENTS

		DOCUMENT NO	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE
A		4,181,6567	Mar 1980	Cassidy	55	37	n. 1983
B		4,481,6397	Nov 1984	Bass	455	68	Nov 14 1984
C							
D							
E							
F							
G							
H							
I							
J							
K							

FOREIGN PATENT DOCUMENTS

		DOCUMENT NO	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS DWG	PP SPEC
L									
M									
N									
O									
P									
Q									

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R	
S	
T	
U	

| EXAMINER | DATE 5/17/59 | |

* A copy of this reference is not being furnished with this office action
(See Manual of Patent Examining Procedure section 707 05 (a).)

EXHIBIT E

| 07/441,189 | 11/22/89 | MORRISON | S | 24302STAN621 |

NISBET, T

EDWARD F. MULLOWNEY
C/O FISH & NEAVE
875 THIRD AVENUE
NEW YORK, NY 10022

185   *10*

09/25/90

FINAL

☒ This application has been examined   ☒ Responsive to communication filed on __11/22/89__ ☒ This action is made final

A shortened statutory period for response to this action is set to expire __THREE__ month(s), _____ days from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned.   35 U.S.C. 133

Part I   THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.   2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449.   4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.   6. ☐ _____

Part II   SUMMARY OF ACTION

1. ☒ Claims _____14-38_____ are pending in the application.

   Of the above, claims _____ are withdrawn from consideration.

2. ☐ Claims _____ have been cancelled.

3. ☐ Claims _____ are allowed.

4. ☒ Claims _____14-38_____ are rejected.

5. ☐ Claims _____ are objected to.

6. ☐ Claims _____ are subject to restriction or election requirement.

7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.

8. ☐ Formal drawings are required in response to this Office action.

9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable. ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).

10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).

11. ☐ The proposed drawing correction, filed on _____ has been ☐ approved. ☐ disapproved (see explanation).

12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____ ; filed on _____

13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

14. ☐ Other

FILE NO _BD-1/QP-7P_
DOCKETED FOR _2-25-92_ BY _____
REFERRED TO _McVeenker_
FISH & NEAVE
875 THIRD AVE. N.Y.C.

The following is a quotation of the first paragraph of 35 U.S.C. 112

"The specification shall contain a written description of the invention and of
the manner and process of making and using it. in such full clear concise and
exact terms as to enable any person skilled in the art to which it pertains or
with which it is most nearly connected to make and use the same and shall set
forth the best mode contemplated by the inventor of carrying out his
invention

Claims 14-38 are rejected under 35 U.S.C. 112. first paragraph. as
enabling only for the claims limited to DNA constructs for expression of a
chimeric polypeptide which is a subunit of an immunoglobulin molecule. See
MPEP 706.03(n) and 706.03 (z). This rejection is maintained for essentially
the same reasons set forth in the previous office action.

The previous examiner held and the present examiner maintains that
the instant application is not enabled for and multi-unit receptor (eg. T-cell
receptors and Major Histocompatibility Complex antigens). There are
essentially three reasons. These multi-chain receptors are not disclosed to
contain constant and variable regions nor are methods disclosed on how to
isolate the DNA in question. Second. not all cell receptors have constant and
variable regions (eg. CD2, CD7. CD20. and Fc). Finally, it would require undo
experimentation to isolate variable and constant region genomic sequences
of these receptors for use ion cross-species chimeric constructions.

Claims 14. 28, 33. and 36 are rejected under 35 U.S.C. 112, first and
second paragraphs, as the claimed invention is not described in such full.
clear. concise and exact terms as to enable any person skilled in the art to
make and use the same. and/or for failing to particularly point out and
distinctly claim the subject matter which applicant regards as the invention.

Claims 14. 28. 33. and 36 are indefinite by claiming constructs for
a multi-unit receptor. cells. and a method for expressing a multi-unit
receptor. Not all multi-unit receptors are taught in the instant invention. Is
immunoglobulin intended rather than the genus multi-unit receptor? If DNA

constructs encoding the supergene family of immunoglobulins. T-cell receptors and MHC antigens was intended. it should be named as such The instant specification does not enable isolation of the DNA for both the constant and variable region genes encoding T-cell receptors or MHC antigens.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office action:

"A person shall be entitled to a patent unless -

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country. more than one year prior to the date of application for patent in the United States."

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent. or on an international application by another who has fulfilled the requirements of paragraphs (1). (2). and (4) of section 371(c) of this title before the invention thereof by the applicant for patent

Claims 14-34. and 36 are rejected under 35 U.S.C. § 102 (b) or 102 (e) as being clearly anticipated by Cabilly (L) or Cabilly (R) or Cabilly (2A).

This rejection is on the same grounds as those of the previous action. All of the above references teach DNA constructs of various chimeric immunoglobulins, subunits or fragments thereof. They also teach expression vectors for these constructs for expression in either eukaryotic or prokaryotic hosts. Cabilly even teaches the expression of the constructs in mammalian cells. In fact, the references anticipate every aspect of the instant application except the specific use of myeloma cells.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title. if the differences between

the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains Patentability shall not be negatived by the manner in which the invention was made

Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title. shall not preclude patentability under this section where the subject matter and the claimed invention were at the time the invention was made. owned by the same person or subject to an obligation of assignment to the same person.

Claims 35, and 37-38 are rejected under 35 U.S.C. 103 as being unpatentable over Cabilly (L) or Cabilly (R) or Cabilly (2A) in view of Gillies (S)

Gillies essentially solves the omission of the Cabilly references discussed supra by teaching the expression of immunoglobin genes in myeloma cells. The application of the Cabilly teaching in view of the Gillies would be obvious to one of ordinary skill in the art. especially given the fact that two of the authors of the Gillies reference are inventors of the instant application. Again. this rejection is essentially a repetition of the rejection of paper 7.

Claims 28-35 are rejected under 35 U.S.C. 103 as being unpatentable over Boss (2B) in view of Gillies (S).

Boss teaches the method of producing antibodies comprising transforming a host cell with DNA sequence encoding each of heavy and light chain of an immunoglobulin. Gillies is applied as above in teaching the use of myeloma cells for expressing heterologous products and the particular advantages of myeloma cells in expressing immunoglobulin genes. For further discussion of the rejection. applicant is referred to the previous action

This is a file wrapper continuation of applicant's earlier application S.N. 07/090,669. All claims are drawn to the same invention claimed in the

earlier application and could have been finally rejected on the grounds or art of record in the next Office action if they had been entered in the earlier application. Accordingly. **THIS ACTION IS MADE FINAL** even though it is a first action in this case. See MPEP 706.07(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). The practice of automatically extending the shortened statutory period an additional month upon the filing of a timely first response to a final rejection has been discontinued by the Office. See 1021 TMOG 35.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED. AND ANY EXTENSION FEE PURSUANT TO 37 CFR 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

An inquiry concerning this communication should be directed to Examiner Nisbet at telephone number 703-557-5137.

RICHARD A. SCHWARTZ
PRIMARY EXAMINER
ART UNIT 124

EXHIBIT F

[54] **RECOMBINANT IMMUNOGLOBIN PREPARATIONS**

[75] Inventors: **Shmuel Cabilly, Monrovia; Herbert L. Heyneker, Burlingame; William E. Holmes, Pacifica; Arthur D. Riggs, La Verne; Ronald B. Wetzel, San Francisco, all of Calif.**

[73] Assignee: **Genentech, Inc., South San Francisco, Calif.**

[21] Appl. No.: **483,457**

[22] Filed: **Apr. 8, 1983**

[51] Int. Cl.⁴ **C07K 15/14; C07K 15/06; C12P 21/00; C12N 15/00; C12N 1/20**

[52] U.S. Cl. **530/387; 435/68; 435/172.3; 435/320; 435/252.3; 435/252.31; 435/252.33; 435/252.34; 935/10; 935/15; 935/29; 935/73; 530/388**

[58] Field of Search .... **435/68, 172.3, 240, 435/253, 172.2, 317, 320; 260/112 B; 536/27; 935/11, 15, 27, 29, 73; 530/387, 388**

[56] **References Cited**

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0073656	8/1982	European Pat. Off.	435/172.3
0068763	1/1983	European Pat. Off. .	
0120694	10/1984	European Pat. Off. .	

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Primary Examiner—Jayme Huleatt

[57] **ABSTRACT**

Altered and native immunoglobulins, including constant-variable region chimeras, are prepared in recombinant cell culture. The immunoglobulins contain variable regions which are immunologically capable of binding predetermined antigens. Methods are provided for refolding directly expressed immunoglobulins into immunologically active form.

7 Claims, 15 Drawing Sheets

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HEAVY CHAIN

LIGHT CHAIN

VARIABLE

VARIABLE

VARIABLE

VARIABLE

$F(ab)_2$ fragment

Fab' fragment

Fc fragment

Fig. 1.
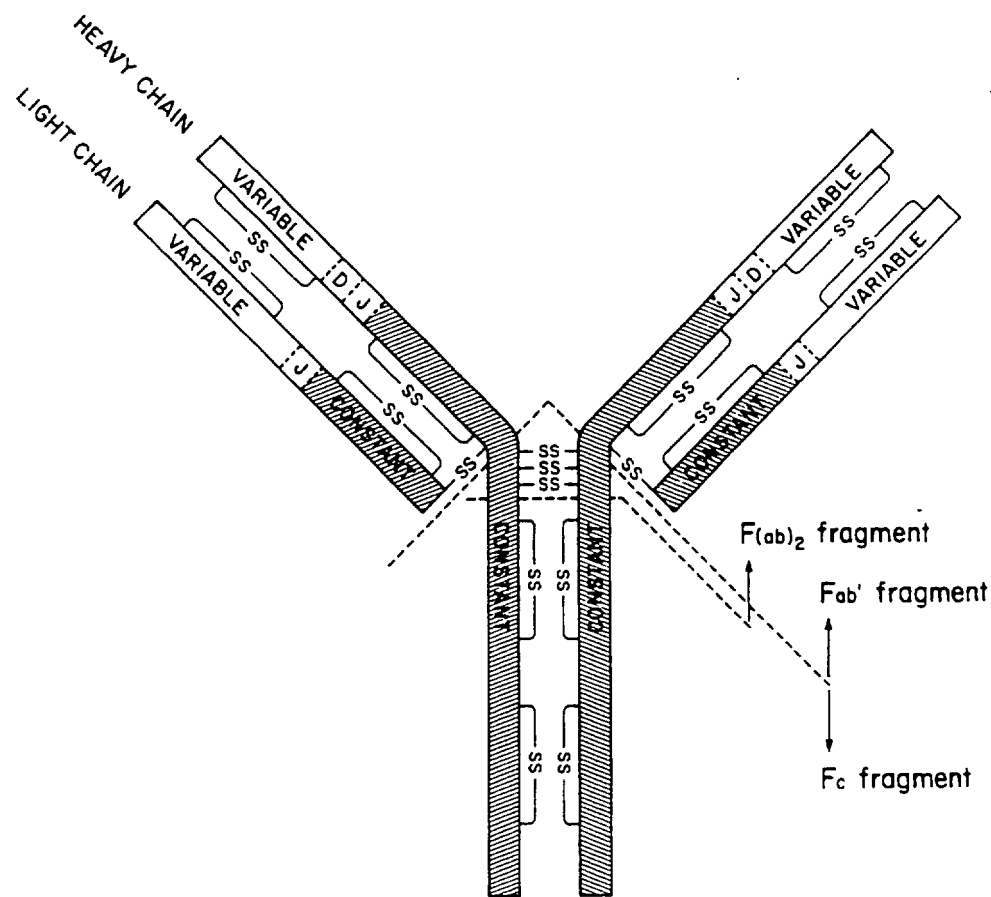
```
                                                          tth111                    hae111
                                                   tth111   hph1                      hae1
1    GTTGCTGTGG TTGTCTGGTG TTGAAGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCAT GTCCACATCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG
     CAACGACACC AACAGACCAC AACTTCCTCT GTAACACTAC TGGGTCAGAG TGTTTAAGTA CAGGTGTAGT CATCCTCTGT CCCAGTCGTA GTGGACGTTC
                                                                                                          sfaNI

              fnu4HI  scrFI                                                              scrFI
        fokI   bbv    ecoRII                                    scrFI                     ncII   hinfI
                                                               ecoRII                     hpaII
101  GCCAGTCAGG ATGTGGGTGC TGCTATAGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTACTGA TTTACTGGGC ATCCACCCGG CACACTGGAG
     CGGTCAGTCC TACACCCACG ACGATATCGG ACCATAGTTG TCTTTGGTCC TGTTAGAGGA TTTGATGACT AAATGACCCG TAGGTGGGCC GTGTGACCTC
                                                                                        fokI   sfaNI

     sau3A
     dpnI                                                      hphI                              hincII
           xhoII
           sau3A
           dpnI
201  TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACAGATTT CACTCTCACC ATTAGCAATG TGCAGTCTGA TGACTTGGCA GATTATTTCT GTCAACAATA
     AGGGACTAGC GAAGTGTCCG TCACCTAGAC CCTGTCTAAA GTGAGAGTGG TAATCGTTAC ACGTCAGACT ACTGAACCGT CTAATAAAGA CAGTTGTTAT

     mnlI                 sau96                     alul sfaNI           fnu4HI                            hpaI
           mnlI           avaII   alul                                  bbv       mboII                   hincII
                                  alul
301  TAGCGGGTAT CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAAC GGGCTGATGC TGCACCAACT GTATCCATCT TCCCACCATC CAGTGAGCAG
     ATCGCCCATA GGAGAGTGCA AGCCACGACC CTGGTTCGAC CTCGACTTTG CCCGACTACG ACGTGGTTGA CATAGGTAGA AGGGTGGTAG GTCACTCGTC
                                                                                            fokI

     mnlI                 xmnI                                          mboII                             acyI
           mnlI
           ddeI
401  TTAACATCTG GAGGTGCCTC AGTTCGTGTGC TTCTTGAACA ACTTCTACCC CAAAGACATC AATGTCAAGT GGAAGATTGA TGGCAGTGAA CGACAAATG
     AATTGTAGAC CTCCACGGAG TCAGCACACG AAGAACTTGT TGAAGATGGG GTTTCTGTAG TTACAGTTCA CCTTCTAACT ACCGTCACTT GCTGTTTAC
```

Fig.2A.

```
                                                                                                    aluI
                                                          fnu4HI
                                                          bbv    mnlI hincII                   GACATAACAG
                                      sau3A                           ATGAGCAGCA  GAGTATGAAC   CTGTATTGTC
                                      dpnI                            CCCTCACGTT  GACCAAGGAC
                                      bcII            GCAAAGACAG CACCTACAGC        CTGGTTCCTG
          hgaI                                                                                         sau96  hgaI
501       GGGTCCTGAA  CAGTTGGACT  GATCAGGACA  GCAAAGACAG  CACCTACAGC  ATGAGCAGCA  GAGTATGAAC   ddeI
          CGCAGGACTT  GTCAACCTGA  CTAGTCCTGT  CGTTTCTGTC  GTGGATGTCG  TACTCGTCGT  GGGAGTGCAA  CTGGTTCCTG  CTCATACTTG  CTGTATTGTC

                                                                                                   avaII   acyI
          mnlI                                                        aluI                          GGTCCTGAGA  CGCCACCACC
          haeIII                      hphI                            GCTTCAACAG  GAATGAGTGT  TAGAGACAAA  GCGGTGGTGG
          haeI                         AACTTCACCC  ATTGTCAAGA                                  CCAGGACTCT
601       CTATACCTGT  GAGGCCACTC  ACAAGACATC  AACTTCACCC  ATTGTCAAGA  GCTTCAACAG  GAATGAGTGT  TAGAGACAAA
          GATATGGACA  CTCCGGTGAG  TGTTCTGTAG  TTGAAGTGGG  TAACAGTTCT  CGAAGTTGTC  CTTACTCACA  ATCTCTGTTT  CCAGGACTCT  GCGGTGGTGG

                           aluI       mboII   ddeI          mnlI                                       mnlI
          aluI             CTCCATCCTA  TCTTCCCTTC  TAAGGTCTTG  GAGGCTTCCC  CACAAGCGAC  CTACCACTGT       mnlI
          AGCTCCCCAG                               GAGGCTTCCC                            GTCGGTGACA  hgIA CAAACCTCCT  CCCCACCTCC
701       AGCTCCCCAG  CTCCATCCTA  TCTTCCCTTC  TAAGGTCTTG  GAGGCTTCCC  CACAAGCGAC  CTACCACTGT  TGCGGTGCTC  CAAACCTCCT  CCCCACCTCC
          TCGAGGGGTC  GAGGTAGGAT  AGAAGGGAAG  ATTCCAGAAC  CTCCGAAGGG  GTGTTCGCTG  GATGGTGACA  ACGCCACGAG  GTTTGGAGGA  GGGGTGGAGG
          fokI

               mnlI
          mnlI  mnlI                                                       hinfI
          TTCTCCTCCT  CCTCCCTTTC  CTTGGCTTTT  ATCATGTGTAA  TATTTGCAGA  TAAAGTGAGT  CTTTGCACTT  GA
801       TTCTCCTCCT  CCTCCCTTTC  CTTGGCTTTT  ATCATGTGTAA  TATTTGCAGA  TAAAGTGAGT  CTTTGCACTT  GA
          AAGAGGAGGA  GGAGGGAAAG  GAACCGAAAA  TAGTACGATT  ATAAACGTCT  TTTATAAGTT  ATTTCACTCA  GAAACGTGAA  CT
```

nucleotides: 882

Fig. 2B.

```
                                   1                              10                          20
      leu leu trp leu ser gly val glu gly val ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser
   G  UUG CUG UGG UUG UCU GGU GUU GAA GGU GUU AUU GUG AUG ACC CAG UCU CAC AAA UUC AUG UCC ACA UCA GUA GGA GAC AGG GUC AGC
     -9

                         30                             40                          50
      ile thr cys lys ser ala lys gln asp gly val gln asp his arg phe thr gly ser gly lys gln gln ... ... lys pro tyr trp
      AUC ACC UGC AAG AGC GCC AAG CAG GAU GGU GUG CAG GAU CAC CGG UUC ACU GGA AGU GGG AAG CAA ... AAG CCA ... UAC UGG

                         60                             70                          80
      ala ser thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp phe thr leu thr ile ser asn val gln ser
      GCA UCC ACC CGG CAC ACU GGU GUC CCU GAU CGC UUC ACU GGU AGU GGA UCU GGG ACA GAU UUC ACU CUC ACC AUU AGC AAU GUG CAG UCU

                         90                            100                         110
      asp asp leu ala asp tyr phe cys gln gln tyr ser leu tyr pro leu thr phe gly gly gly thr lys leu glu leu lys arg ala asp
      GAU GAC CUG GCA GAU UAU UUC UGU CAA CAA UAU AGU CUC UAU CCU CUC ACG UUC GGU GGU GGG ACG AAG CUG GAG CUG AAA CGG GCU GAU

                        120                            130                         140
      ala ala pro thr val ser ile phe pro pro ser ser glu gln leu thr ser gly gly ala ser val val cys phe leu asn asn phe tyr
      GCU GCA CCA ACU GUA UCC AUC UUC CCA CCA UCC AGU GAG CAG UUA ACA UCU GGA GGU GCC UCA GUC GUG UGC UUC UUG AAC AAC UUC UAC

                        150                            160                         170
      pro lys asp ile asn val lys trp lys ile asp gly ser glu arg gln asn gly val leu asn ser trp thr asp gln asp ser lys asp
      CCC AAA GAC AUC AAU GUC AAG UGG AAG AUU GAU GGC AGU GAA CGA CAA AAU GGC GUC CUG AAC AGU UGG ACU GAU CAG GAC AGC AAA GAC

                        180                            190                         200
      ser thr tyr ser met ser ser thr leu thr leu thr lys asp glu tyr glu arg his asn ser tyr thr cys glu ala thr his lys thr
      AGC ACC UAC AGC AUG AGC AGC ACC CUC ACG UUG ACC AAG GAC GAA UAU GAG CGA CAU AAC AGC UAU ACU UGU GAG GCC ACU CAC AAG ACA

                        210                214 AM
      ser thr ser pro ile val lys ser phe asn arg asn glu cys AM
      UCA ACU UCA CCC AUU GUC AAG AGC UUC AAC AGG AAU GAG UGU UAG   AGACAAAGGUUCUGAGUCCAGCUCCACCACCAGCUCCUCCACCUCCUCCUCCCUUUCCUUCCUUGGCUUUUAUCAUGGCUAAAUUUGCAGAAAA

   GGUCUUGGAGGAGGCUUCCCACCACAAGCGACCUACCACUGUCCCACUGUCGGCUCCAAACCACCUCCCACCUCCUCCUCCCUUUCCCUUCCUUGGCUUUUAUCAUGGCUAAAUUUGCAGAAAA

   UAUUCAAUAAAGUGAGUGAGUCUUUGCACUUGA
```

Fig. 3.

Fig. 4A.

```
                                                                                                                        xhoII
                                                                                                                        sau3A
                                                              scrFI                                        scrFI        dpnI
                                          ncoI  sfaNI  fokI   ecoRII                          fnu4HI        ecoRII       bamHI
                                          hphI  ecoRII        scrFI                  pvuII    bbv  ddeI                         sau96
                                          bstEII                            ddeI     aluI          mnlI           mnlI  haeIII       hphI
     fnu4HI                         scrFI                                            pstI   mnlI
     bbv                            hphI  ecoRII
501  GCTGCCCAAA CTAACTCCAT GGTGACCCTG GGATGCCCTG TCAAGGGCTA TTTCCCTGAG CCAGTGACAG CTCTGGATCC CTGTCCAGCG
     CGACGGGTTT GATTGAGGTA CCACTGGGAC CCTACGGGAC AGTTCCCGAT AAAGGGACTC GGTCACTGTC GAGACCTAGG GACAGGTCGC

         scrFI
         haeIII
         ncII  fnu4HI
     hgfA      bbv
     aluI
601  GTGTGCACAC CTTCCCAGCT GTCTGCAGT CTGACCTCTA CACTCTGAGC AGCTCAGTGA CTGTCCCCTC CAGCCCTCGG CCCAGCCGAGA CCGTCACCTG
     CACAGCGTGTG GAAGGGTCGA CAGGAGTCA GACTGGAGAT GTGAGACTCG TCGAGTCACT GACAGGGGAG GTCGGGAGCC GGGTCGGCTCT GGCAGTGGAC

         bglI  hpaII                                                    scrFI                  ndeI  rsaI
     CAACGTTGCC CACCCGGCCA GCAGCACCAA GGTGGACAAG AAAATTGTGC CCAGGGATTG   ecoRII                 GTACAGTCCC AGAAGTATCA
701  GTTGCAACGG GTGGGCCGGT CGTCGTGGTT CCACCTGTTC TTTTAACACG GGTCCCTAAC  CCTTGTGTAAG            CATGTCAGGG TCTTCATAGT
                                                                         CCTTGCATAT
                                                                         GGAACGTATA

                          fokI  hgfA                            mstII            accI
                          mboII                  hinfI          ddeI
801  TCTGTCTTCA TCTTCCCCCC AAAGCCCAAG GATGTGCTCA CCATTACTCT GACTCCTAAG GTCACGTGTG TTGTGGTAGA CATCAGCAAG GATGATCCCG
     AGACAGAAGT AGAAGGGGGG TTTCGGGTTC CTACACGAGT GGTAATGAGA CTGAGGATTC CAGTGCACAC AACACCATCT GTAGTCGTTC CTACTAGGGC

                                              smaI
                                              scrFI
                                              scrFI
                                              ncII
                                              ncII
                    ddeI  aluI  hgaI          hpaII  mnlI
     sau96  pvuII   mnlI        aluI  hgaI    aval
     avaII  aluI
901  AGGTCCAGTT CAGCTGGTTT GTAGATGATG TGGAGGTGCA CACAGTCAG ACGCAACCCC GGGAGGAGGCA GTTCAACAGC ACTTTCCGCT CAGTCAGTGA
     TCCAGGTCAA GTCGACCAAA CATCTACTAC ACCTCCACGT GTGTCAGTC TGCGTTGGGG CCCTCCTCGT CAAGTTGTCG TGAAAGGCGA GTCAGTCACT
```

Fig. 4B.

```
                                                          fnu4HI
                                                          bbv        aluI                        taqI
              scrFI
              ecoRII                             hincII                                          
1001  ACTTCCATC ATGCACCAGG ACTGGCTCAA TGGCAAGGAG TTCAAATGCA GGGTCAACAG TGCAGCTTTC CCTGCCCCCA TCGAGAAAAC CATCTCCAAA
      TGAAGGGTAG TACGTGGTCC TGACCGAGTT ACCGTTCCTC AAGTTTACGT CCCAGTTGTC ACGTCGAAAG GGACGGGGGT AGCTCTTTTG GTAGAGGTT

                                     haeIII
                                     hael
                                     ball
              rsal          mnll
1101  ACCAAAGGCA GACCGAAGGC TCCACAGGTG TACACCATTC CACCTCCCAA GGAGCAGATG GCCAAGGATA AAGTCAGTCT GACCTGCATG ATAACAGACT
      TGGTTTCCGT CTGGCTTCCG AGGTGTCCAC ATGTGGTAAG GTGGAGGGTT CCTCGTCTAC CGGTTCCTAT TTCAGTCAGA CTGGACGTAC TATTGTCTGA

      mboll mboll             fnu4HI                     ddel
                              bbv
1201  TCTTCCCTGA AGACATTACT GTGGAGTGGC AGTGGAATGG GCAGCCAGCG GAGAACTACA AGAACACTCA GCCCATCATG AACACGAATG GCTCTTACTT
      AGAAGGGACT TCTGTAATGA CACCTCACCG TCACCTTACC CGTCGGTCGC CTCTTGATGT TCTTGTGAGT CGGGTAGTAC TTGTGCTTAC CGAGAATGAA

                                                                                 sau96
                                                                                 mnll          mboll
      accI    alul     mboll       mnll            hphI    haelll               ddel
1301  CGTCTACAGC AAGGTCAATG TGCAGAAGAG CAACTGGGAG GCAGGAAATA CTTTCACCTG CTCTGTGTTA CATGAGGGCC TGCACAACCA CCATACTGAG
      GCAGATGTCG TTCGAGTTAC ACGTCTTCTC GTTGACCCTC CGTCCTTTAT GAAAGTGGAC GAGACACAAT GTACTCCCGG ACGTGTTGGT GGTATGACTC

      scrFI        sau3A                    mnll  avall
      ecoRII       dpnl           mnll       hinfl                mnll
1401  AAGAGCCTCT CCCACTCTCC TGGTAAATGA TCCCAGTGTC CTTGGAGCCC TCTGGTCCTA CAGGACTCTG ACACCTACCT CCACCCCTCC CTGTATAAAT
      TTCTCGGAGA GGGTGAGAGG ACCATTTACT AGGGTCACAG GAACCTCGGG AGACCAGGAT GTCCTGAGAC TGTGGATGGA GGTGGGGAGG GACATATTTA

1501  AAGCACCCCA GCACTGCCTT GGGAAAAAA
      TTCGTGGGGT CGTGACGGAA CCCTTTTT
```
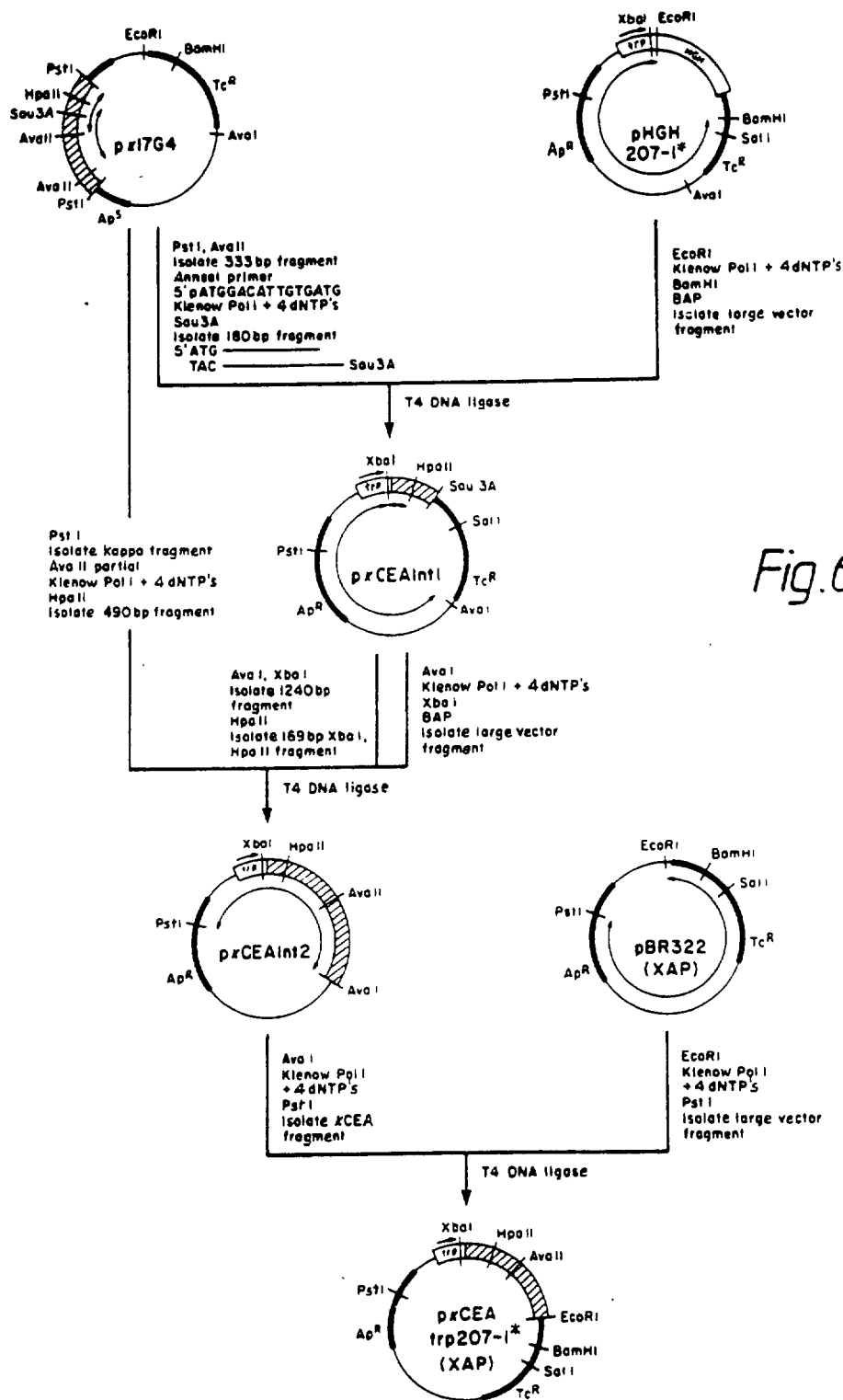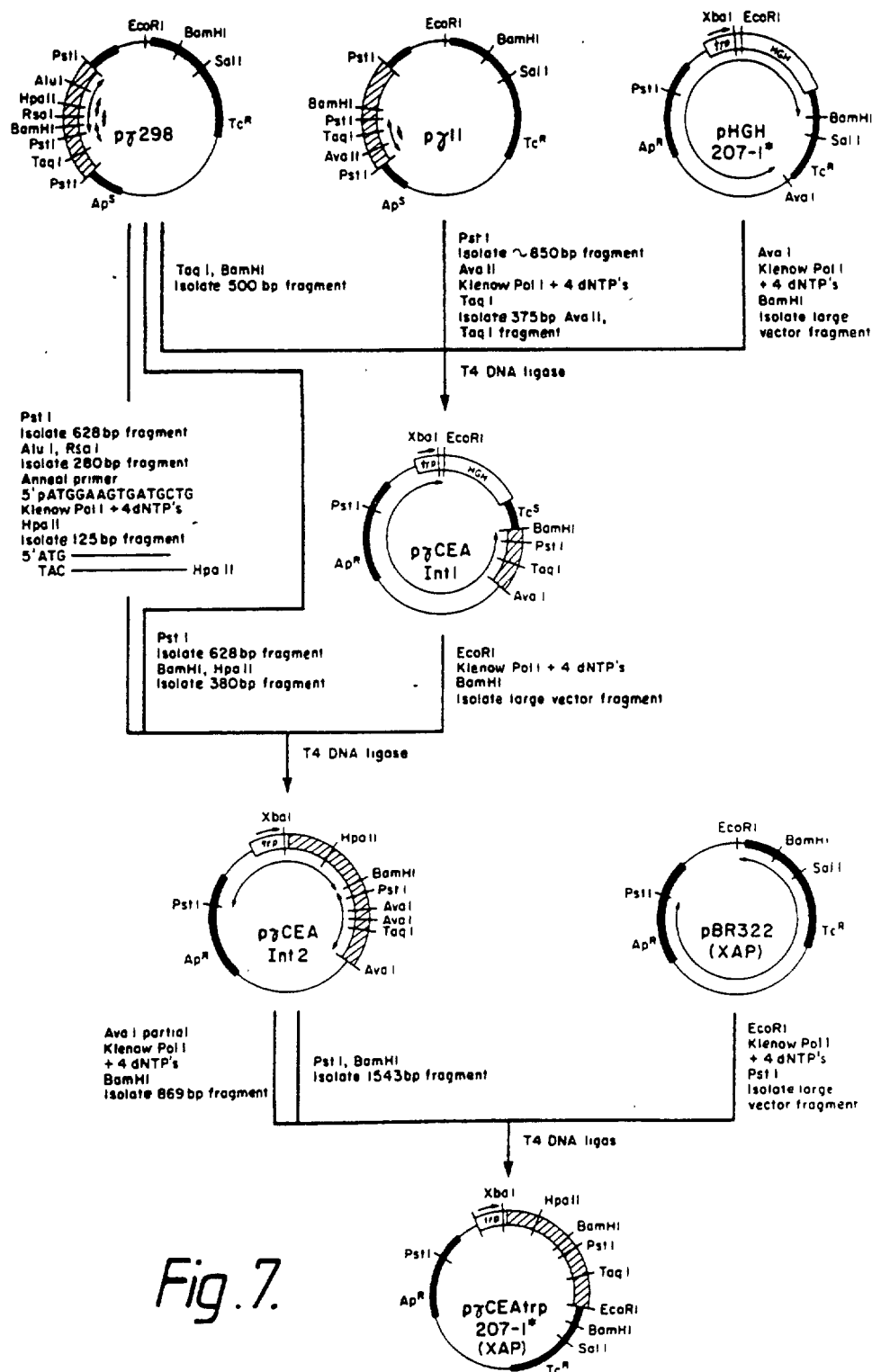
Fig. 4C.

```
GAGUCAGCACUGAACACGGACCCCUCACG
                                                                      -10                                            1
              met asn phe gly ser leu ile tyr   leu val leu val leu leu lys val   val gln cys glu
              AUG AAC UUC GGG CUC UUG AUU UAC   CUU GUC CUG GUU UUA AAA GUU GUC   CAG UGU GAA

         10                                20                                30
val met leu val glu ser gly gly   val leu val gln pro gly gly ser   leu lys leu ser cys ala ala ser gly phe thr phe   ser arg
GUG AUG CUG GUG GAG UCU GGG GGA   GUU UUA GUC CAG CCU GGA GGG UCU   UUG AAA CUC UCA UGU GCC GCC UCU GGA UUU ACU UUC   AGU AGA

         40                                50                                60
tyr ala met ser trp val arg gln thr pro glu lys arg leu glu trp val   ile thr ser ser ile ser   ser his leu pro ser
UAU GCC AUG UCU UGG GUU CGC CAG ACU CCG GAG AAG AGG CUG GAG UGG GUC   AUU ACC AGU AGU AUC AGU   UCA CAC CUU CCA UCC

         70                                80                                90
arg gln cys glu gly phe thr phe phe ile ser arg asp asn ala lys asn thr leu tyr leu gln met ser   ser leu arg ser glu asp thr
AGA CAG UGU GAA GGG CGA UUC ACC AUC UCC AGA GAC AAU GCC AAG AAC ACC CUG UAC CUG CAA AUG AGC   AGC CUG AGG UCU GAG GAC ACG

        100                               110                               120
ala met tyr tyr cys ala arg pro   ile ser leu val ala asp tyr tyr   gln gly thr trp met ser   val thr ser val thr val
GCC AUG UAU UAC UGU GCA AGA CCC   AUU UCG CUU GUA GCG GAC UAU UAU   CAA GGU UGG AUG UCC   GUC UCA ACC GUC ACC

        130                               140                               150
ser ala thr lys thr thr pro pro   leu ala leu ser ala ala ala ser   met val thr leu gly cys leu
UCA GCA ACA AAA ACG ACA CCC CCA   CUG GCC CUG UCG GCC GCC UCC   AUG GUG ACC CUG GGA UGC CUG

        160                               170                               180
val lys gly tyr phe pro phe pro   trp asn ser gly ser ser val   his thr phe pro ala val leu gln
GUC AAG GGC UAU UUC CCU UUC CCA   UGG AAC UCU GGA UCC AGC GUG   CAC ACC UUC CCU GCU GUC CUG CAG

        190                               200                               210
ser ser ser leu thr val thr   ser ser pro ser ser arg pro ser   asn cys lys val asn his lys pro ala
AGC UCA AGC CUC ACU GUC ACU   AGC UCC CCA UCC UCU AGG CCG UCU   AAC UGC AAG GUU AAC CAC AAG CCG GCC

        220                               230                               240
ser thr lys lys val asp asp cys gly cys lys pro   ile cys thr glu pro glu   ser ser val phe
AGC ACC AAG AAG GUG GAC GAU UGU GGU UGU AAG CCU   AUA UGC ACA GAA CCA GAA   UCA UCU GUC UUC
```

Fig. 5A.

```
        ile phe pro lys pro lys asp   val leu thr ile thr leu thr pro lys val   thr cys val val asp ile ser lys asp   pro
        AUC UUC CCC AAG CCA AAG GAU   GUG CUC ACC AUU ACU CUG ACU CCU AAG GUC   ACG UGU GUU GUA GAC AUC AGC AAG GAU   CCC
                                      250                                       260                                   270

        glu val gln phe ser trp phe val asp   asp val glu val his thr ala gln thr   gln pro arg glu glu gln phe asn ser   thr phe arg
        GAG GUC CAG UUC AGC UGG UUU GUA GAU   GAC GUG GAG GUG CAC ACA GCU CAG ACG   CAG CCC CGG GAG GAG CAG UUC AAC AGC   ACU UUC CGC
                                              280                                  290                                  300

        ser val ser glu leu pro ile met   his gln asp trp leu asn gly lys glu phe   lys cys arg val asn ser ala ala phe   pro ala pro
        UCA GUC AGU GAA CUU CCC AUC AUG   CAC CAG GAC UGG CUC AAU GGC AAG GAG UUC   AAA UGC AGG GUC AAC AGC GCU GCA UUC   CCU GCA CCC
                                          310                                      320                                  330

        ile glu lys thr ile ser lys thr   lys gly arg pro lys ala pro gln val   tyr thr ile pro pro pro lys glu gln met   ala lys asp
        AUC GAG AAA ACC AUC UCC AAA ACC   AAA GGC AGA CCG AAG GCU CCA CAG GUC   UAC ACU AUU CCU CCA CCC AAG GAG CAG AUG   GCC AAG GAU
                                          340                                   350                                      360

        lys val ser leu thr cys met ile   thr asp phe phe pro glu asp ile thr val   glu trp gln trp asn gly gln pro ala   glu asn tyr
        AAA GUC AGU CUG ACC UGC AUG AUA   ACA GAC UUC UUC CCU GAA GAC AUC ACU GUG   GAG UGG CAG UGG AAU GGG CAG CCA GCG   GAG AAC UAC
                                          370                                      380                                  390

        lys asn thr gln pro ile met asp   thr asp gly ser tyr phe val tyr ser lys   leu asn val gln lys ser asn trp glu   gly ala asn
        AAG AAC ACG CAG CCC AUC AUG GAU   ACA GAC GGU UCA UAC UUC GUC UAC AGC AAG   CUC AAU GUU CAG AAG AGC AAC UGG GAG   GGA GCA AAU
                                          400                                      410                                  420

        thr phe thr cys ser val leu his glu gly   leu his asn his his thr glu lys ser leu   ser his ser pro gly lys OP
        ACU UUC ACC UGC UCU GUG UUA CAU GAG GGC   CUG CAC AAC CAC CAC ACU GAG AAG AGC CUC   UCC CAC UCC CCU GGU AAA UGA   UCCCAGUGUCCU
                                                 430                                      440                           447

        UGGAGCCCUCUGGUCCUUCCAGGACCUACUGACUCUGAGACACCUACCUCCUCCCCUGUAUAAAAAAGCACCACCCAGCACUGCCUUGGGAAAAA
```

Fig. 5B.

Fig.6.

Fig.7.

Fig. 8A.

Fig. 8B.

Fig. 8C.

30 ng	70 ng	0.10 μg anti-CEA
90 ng	210 ng	0.30 μg anti-CEA
300 ng	700 ng	1.00 μg anti-CEA

K cells (10 μl)

γ cells (10 μl)

K γ cells (10 μl)

RW57-K3000

RW57-K3000

$\left. \begin{array}{l} 31-35 \\ 31-35 \end{array} \right\}$ K-G75

K γ cells (20 μl)

K cells (20 μl)

Fig.9.

Fig. 10.

Fig. 11.

DELETE AS FOLLOWS:

- Clone *Xba*I to *Pvu*II fragment in M13

- *In vitro* site-directed deletion mutagenesis

- Clone modified *Xba*I to *Pvu*II fragment back in p-Intermediate human/mouse hybrid (XAP)

Fig.12.

Fig. 13.

1

RECOMBINANT IMMUNOGLOBIN PREPARATIONS

BACKGROUND OF THE INVENTION

This invention relates to the field of immunoglobulin production and to modification of naturally occurring immunoglobulin amino acid sequences. Specifically, the invention relates to using recombinant techniques to produce both immunoglobulins which are analogous to those normally found in vertebrate systems and to take advantage of these gene modification techniques to construct chimeric or other modified forms.

A. Immunoglobulins and Antibodies

Antibodies are specific immunoglobulin polypeptides produced by the vertebrate immune system in response to challenge by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is remarkable in its complexity and variability. Thousands of antigens are capable of eliciting responses, each almost exclusively directed to the particular antigen which elicited it.

Immunoglobulins include both antibodies, as above described, and analogous protein substances which lack antigen specificity. The latter are produced at low levels by the lymph system and in increased levels by myelomas.

A.1 Source and Utility

Two major sources of vertebrate antibodies are presently utilized—generation in situ by the mammalian B lymphocytes and in cell culture by B-cell hybrids. Antibodies are made in situ as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cell, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are reassembled sequentially prior to transcription. A review of this process has been given by Gough, *Trends in Biochem Sci*, 6: 203 (1981). The resulting rearranged genome is capable of expression in the mature B lymphocyte to produce the desired antibody. Even when only a single antigen is introduced into the sphere of the immune system for a particular mammal, however, a uniform population of antibodies does not result. The in situ immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibody is contributed by a single population of B cells—hence in situ generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies (Kohler, et al., *Eur. J. Immunol.*, 6: 511 (1976)). In this process, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with a tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell.

2

The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce immunoreactive antibodies against a desired antigen which are assured to be homogenous, and which antibodies, referencing their pure genetic parentage, are called "monoclonal". Hybridoma technology has to this time been focused largely on the fusion of murine lines, but human-human hybridomas (Olsson, L. et al., *Proc. Natl. Acad. Sci. (USA)*, 77: 5429 (1980)); human-murine hybridomas (Schlom, J., et al. (ibid) 77: 6841 (1980)) and several other xenogenic hybrid combinations have been prepared as well. Alternatively, primary, antibody producing, B cells have been immortalized in vitro by transformation with viral DNA.

Polyclonal, or, much more preferably, monoclonal, antibodies have a variety of useful properties similar to those of the present invention. For example, they can be used as specific immunoprecipitating reagents to detect the presence of the antigen which elicited the initial processing of the B cell genome by coupling this antigen-antibody reaction with suitable detection techniques such as labeling with radioisotopes or with enzymes capable of assay (RIA, EMIT, and ELISA). Antibodies are thus the foundation of immuno diagnostic tests for many antigenic substances. In another important use, antibodies can be directly injected into subjects suffering from an attack by a substance or organism containing the antigen in question to combat this attack. This process is currently in its experimental stages, but its potential is clearly seen. Third, whole body diagnosis and treatment is made possible because injected antibodies are directed to specific target disease tissues, and thus can be used either to determine the presence of the disease by carrying with them a suitable label, or to attack the diseased tissue by carrying a suitable drug.

Monoclonal antibodies produced by hybridomas, while theoretically effective as suggested above and clearly preferable to polyclonal antibodies because of their specificity, suffer from certain disadvantages. First, they tend to be contaminated with other proteins and cellular materials of hybridoma, (and, therefore, mammalian) origin. Second, hybridoma lines producing monoclonal antibodies tend to be unstable and may alter the structure of antibody produced or stop producing antibody altogether (Kohler, G., et al., *Proc. Antl. Acad. Sci (USA)* 77: 2197 (1980); Morrison, S. L., *J. Immunol.* 123: 793 (1979)). The cell line genome appears to alter itself in response to stimuli whose nature is not currently known, and this alteration may result in production of incorrect sequences. Third, both hybridoma and B cells inevitably produce certain antibodies in glycosylated form (Melchers, F., *Biochemistry*, 10: 653 (1971)) which, under some circumstances, may be undesirable. Fourth, production of both monoclonal and polyclonal antibodies is relatively expensive. Fifth, and perhaps most important, production by current techniques (either by hybridoma or by B cell response) does not permit manipulation of the genome so as to produce antibodies with more effective design components than those normally elicited in response to antigens from the mature B cell in situ. The antibodies of the present invention do not suffer from the foregoing drawbacks, and, furthermore, offer the opportunity to provide molecules of superior design.

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Even those immunoglobulins which lack the specificity of antibodies are useful, although over a smaller spectrum of potential uses than the antibodies themselves. In presently understood applications, such immunoglobulins are helpful in protein replacement therapy for globulin related anemia. In this context, an inability to bind to antigen is in fact helpful, as the therapeutic value of these proteins would be impaired by such functionality. At present, such non-specific antibodies are derivable in quantity only from myeloma cell cultures suitably induced. The present invention offers an alternative, more economical source. It also offers the opportunity of cancelling out specificity by manipulating the four chains of the tetramer separately.

A.2 General Structure Characteristics

The basic immunoglobin structural unit in vertebrate systems is now well understood (Edelman, G. M., *Ann. N.Y. Acad. Sci.*, 190: 5 (1971)). The units are composed to two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the divergent region as shown in FIG. 1. The "branch" portion, as there indicated, is designated the Fab region. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them, and the nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE. Light chains are classified as either kappa or lambda. Each heavy chain class can be prepared with either kappa or lambda light chain. The light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if noncovalent association of the chains can be effected in the correct geometry, the aggregate will still be capable of reaction with antigen, or of utility as a protein supplement as a non-specific immunoglobulin.

The amino acid sequence runs from the N-terminal end at the top of the Y to the C-terminal end at the bottom of each chain. At the N-terminal end is a variable region which is specific for the antigen which elicited it, and is approximately 100 amino acids in length, there being slight variations between light and heavy chain and from antibody to antibody. The variable region is linked in each chain to a constant region which extends the remaining length of the chain. Linkage is seen, at the genomic level, as occuring through a linking sequence known currently as the "J" region in the light chain gene, which encodes about 12 amino acids, and as a combination of "D" region and "J" region in the heavy chain gene, which together encode approximately 25 amino acids.

The remaining portions of the chain are referred to as constant regions and within a particular class do not to vary with the specificity of the antibody (i.e., the antigen eliciting it).

As stated above, there are five known major classes of constant regions which determine the class of the immunoglobulin molecule (IgG, IgM, IgA, IgD, and IgE corresponding to γ, μ, α, δ, and ϵ heavy chain constant regions). The constant region or class determines subsequent effector function of the antibody, including activation of complement (Kabat, E. A.,

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Structural Concepts in Immunology and Immunochemistry, 2nd Ed., p. 413-436, Holt, Rinehart, Winston (1976)), and other cellular responses (Andrews, D. W., et al., *Clinical Immunobiology*, pp 1-18, W. B. Sanders (1980); Kohl, S., et al., *Immunology*, 48: 187 (1983)); while the variable region determines the antigen with which it will react.

B. Recombinant DNA Technology

Recombinant DNA technology has reach sufficient sophistication that it includes a repertoire of techniques for cloning and expression of gene sequences. Various DNA sequences can be recombined with some facility, creating new DNA entities capable of producing heterologous protein product in transformed microbes and cell cultures. The general means and methods for the in vitro ligation of various blunt ended or "sticky" ended fragments of DNA, for producing expression vectors, and for transforming organisms are now in hand.

DNA recombination of the essential elements (i.e., an origin of replication, one or more phenotypic selection characteristics, expression control sequence, heterologous gene insert and remainder vector) generally is performed outside the host cell. The resulting recombinant replicable expression vector, or plasmid, is introduced into cells by transformation and large quantities of the recombinant vehicle is obtained by growing the transformant. Where the gene is properly inserted with reference to portions which govern the transcription and translation of the encoded DNA message, the resulting expression vector is useful to produce the polypeptide sequence for which the inserted gene codes, a process referred to as "expression." The resulting product may be obtained by lysis, if necessary, of the host cell and recovery of the product by appropriate purifications from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment.

The art of maintaining cell or tissue cultures as well as microbial systems for studying genetics and cell physiology is well established. Means and methods are available for maintaining permanent cell lines, prepared by successive serial transfers from isolated cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems.

SUMMARY OF THE INVENTION

The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. These antibodies and NSIs can be readily prepared in pure "monoclonal" form. They can be manipulated at the genomic level to produce chimeras of variants which draw their homology from species which differ from each other. They can also be manipulated at the protein level, since all four chains do not need to be produced by the same cell. Thus, there are a number of "types" of immunoglobulins encompassed by the invention.

First, immunoglobulins, particularly antibodies, are produced using recombinant techniques which mimic

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the amino acid sequence of naturally occuring antibodies produced by either mammalian B cells in situ, or by B cells fused with suitable immortalizing tumor lines, i.e., hybridomas. Second, the methods of this invention produced, and the invention is directed to, immunoglobulins which comprise polypeptides not hitherto found associated with each other in nature. Such reassembly is particularly useful in producing "hybrid" antibodies capable of binding more than one antigen; and in producing "composite" immunoglobuins wherein heavy and light chains of different origins essentially damp out specificity. Third, by genetic manipulation, "chimeric" antibodies can be formed wherein, for example, the variable regions, correspond to the amino acid sequence from one mammalian model system, whereas the constant region mimics the amino acid sequence of another. Again, the derivation of these two mimicked sequences may be from different species. Fourth, also by genetic manipulation, "altered" antibodies with improved specificity and other characteristics can be formed.

Two other types of immunoglobulin-like moieties may be produced: "univalent" antibodies, which are useful as homing carriers to target tissues, and "Fab proteins" which include only the "Fab" region of an immunoglobulin molecule i.e, the branches of the "Y". These univalent antibodies and Fab fragments may also be "mammalian" i.e., mimic mammalian amino acid sequences; novel assemblies of mammalian chains, or chimeric, where for example, the constant and variable sequence patterns may be of different origin. Finally, either the light chain or heavy chain alone, or portions thereof, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

In other aspects, the invention is directed to DNA which encodes the aforementioned NSIs, antibodies, and portions thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the host cells and cell cultures which result from transformation with these vectors. Finally, the invention is directed to methods of producing these NSIs and antibodies, and the DNA sequences, plasmids, and transformed cells intermediate to them.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a representation of the general structure of immunoglobulins.

FIG. 2A and 2B show the detailed sequence of the cDNA insert of pK17G4 which encodes kappa anti CEA chain.

FIG. 3 shows the coding sequence of the fragment shown in FIG. 2, along with the corresponding amino acid sequence.

FIGS. 4A, 4B and 4C show the combined detailed sequence of the cDNA inserts of pγ298 and pγ11 which encode gamma anti CEA chain.

FIGS. 5A and 5B show the corresponding amino acid sequence encoded by the fragment in FIG. 4.

FIGS. 6 and 7 outline the construction of expression vectors for kappa and gamma anti-CEA chains respectively.

FIGS. 8A, 8B, and 8C show the results of sizing gels run on extracts of E. coli expressing the genes for gamma chain, kappa chain, and both kappa and gamma chains respectively.

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FIG. 9 shows the results of western blots of extracts of cells transformed as those in FIGS. 8.

FIG. 10 shows a standard curve for ELISA assay of anti CEA activity.

FIGS. 11 and 12 show the construction of a plasmid for expression of the gene encoding a chimeric heavy chain.

FIG. 13 shows the construction of a plasmid for expression of the gene encoding the Fab region of heavy chain.

DETAILED DESCRIPTION

A. Definitions

As used herein, "antibodies" refers to tetramers or aggregates thereof which have specific immunoreactive activity, comprising light and heavy chains usually aggregated in the "Y" configuration of FIG. 1, with or without covalent linkage between them; "immunoglobulins" refers to such assemblies whether or not specific immunoreactive activity is a property. "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess specificity—i.e., those which are not antibodies.

"Mammalian antibodies" refers to antibodies wherein the amino acid sequences of the chains are homologous with those sequences found in antibodies produced by mammalian systems, either in situ, or in hybridomas. These antibodies antibodies mimic antibodies which are otherwise capable of being generated, although in impure form, in these traditional systems.

"Hybrid antibodies" refers to antibodies wherein chains are separately homologous with reference mammalian antibody chains and represent novel assemblies of them, so that two different antigens are precipitable by the tetramer. In hybrid antibodies, one pair of heavy and light chain is homologous to antibodies raised against one antigen, while the other pair of heavy and light chain is homologous to those raised against another antigen. This results in the property of "divalence" i.e., ability to bind two antigens simultaneously Such hybrids may, of course, also be formed using chimeric chains, as set forth below.

"Composite" immunoglobulins means those wherein the heavy and light chains mimic those of different species origins or specificities, and the resultant is thus likely to be a non-specific immunoglobulin (NSI), i.e.— lacking in antibody character.

"Chimeric antibodies" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chains is homologous to corresponding sequences in another. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to the sequences in antibodies derived from another. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be derived from presently known sources using readily available hybridomas or B cells from non human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation, and the specificity is not affected by its source, the constant region being human, is less likely to elicit an immune response from

a human subject, when the antibodies are injected than would the constant region from a non-human source.

However, the definition is not limited to this particular example. It includes any antibody in which either or both of the heavy or light chains are composed of combinations of sequences mimicking the sequences in antibodies of different sources, whether these sources be differing classes, differing antigen responses, or differing species of origin and whether or not the fusion point is at the variable/constant boundary. Thus, it is possible to produce antibodies in which neither the constant nor the variable region mimic known antibody sequences. It then becomes possible, for example, to construct antibodies whose variable region has a higher specific affinity for a particular antigen, or whose constant region can elicit enhanced complement fixation or to make other improvements in properties possessed by a particular constant region.

"Altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a mammalian or other vertebrate antibody. Because of the relevance of recombinant DNA techniques to this invention, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the constant region. Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics. The antibody can also be engineered so as to aid the specific delivery of a toxic agent according to the "magic bullet" concept. Alterations, can be made by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques (Dalbadie-McFarland, et al Proc. Natl. Acad. Sci.(USA), 79:6409 (1982)).

"Univalent antibodies" refers to aggregations which comprise a heavy chain/light chain dimer bound to the Fc (or stem) region of a second heavy chain. Such antibodies are specific for antigen, but have the additional desirable property of targeting tissues with specific antigenic surfaces, without causing its antigenic effectiveness to be impaired—i.e., there is no antigenic modulation. This phenomenon and the property of univalent antibodies in this regard is set forth in Glennie, M. J., et al., Nature, 295: 712 (1982). Univalent antibodies have heretofore been formed by proteolysis.

"Fab" region refers to those portions of the chains which are roughly equivalent, or analogous, to the sequences which comprise the Y branch portions of the heavy chain and to the light chain in its entirety, and which collectively (in aggregates) have been shown to exhibit antibody activity. "Fab protein", which protein is one of the aspects of the invention, includes aggregates of one heavy and one light chain (commonly known as Fab'), as well as tetramers which correspond to the two branch segments of the antibody Y, (commonly known as F(ab)₂), whether any of the above are covalently or non-covalently aggregated, so long as the aggregation is capable of selectively reacting with a particular antigen or antigen family. Fab antibodies have, as have univalent ones, been formed heretofore by proteolysis, and share the property of not eliciting antigen modulation on target tissues. However, as they lack

the "effector" Fc portion they cannot effect, for example, lysis of the target cell by macrophages.

"Fab protein" has similar subsets according to the definition of the present invention as does the general term "antibodies" or "immunoglobulins". Thus, "mammalian" Fab protein, "hybrid" Fab protein "chimeric", Fab and "altered" Fab protein are defined analogously to the corresponding definitions set forth in the previous paragraphs for the various types of antibodies.

Individual heavy or light chains may of course be "mammalian", "chimeric" or "altered" in accordance with the above. As will become apparent from the detailed description of the invention, it is possible, using the techniques disclosed to prepare other combinations of the four-peptide chain aggregates, besides those specifically defined, such as hybrid antibodies containing chimeric light and mammalian heavy chains, hybrid Fab proteins containing chimeric Fab proteins of heavy chains associated with mammalian light chains, and so forth.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Clearly a lack of replicability would render them effectively inoperable. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence—i.e. a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. As at present, such vectors are frequently in the form of plasmids, thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time become known in the art.

"Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

B. Host Cell Cultures and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example, E. coli K12 strain

294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* B, and *E. coli* X1776 (ATTC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F³¹, λ⁻, prototrophic, ATTC No. 27325), bacilli such as *Bacillus subtilus* and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al, *Nature* 275: 615 (1978); Itakura, et al, *Science*, 198: 1056 (1977); (Goeddel, et al *Nature* 281: 544 (1979)) and a tryptophan (trp) promoter system (Goeddel, et al, *Nucleic Acids Res*, 8: 4057 (1980); EPO Appl Publ No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist, et al, *Cell* 20: 269 (1980)).

In addition to prokaryates, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb, et al, *Nature* 282: 39 (1979); Kingsman et al, *Gene*, 7: 141 (1979); Tschemper et al, *Gene*, 10: 157 (1980)) is commonly used. This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12 (1977)). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.*, 255: 2073 (1980)) or other glycolytic enzymes (Hess, et al, *J. Adv. Enzyme Reg.*, 7: 149 (1968); Holland, et al, *Biochemistry*, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, an enzymes responsible for maltose and galactose utilization (Holland, ibid.). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However interest has been greatest in vertebrate cells, and propogation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers. et al, *Nature*, 273: 113 (1978)) incorporated herein by reference. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g. Polyoma, Adeno, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to those host cells, vectors and expression systems exemplified.

C. Methods Employed

C.1 Transformation

If cells without formidable cell wall barriers are used as host cells, transfection is carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52: 546 (1978). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method

of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al *Proc. Natl. Acad. Sci. (USA)*, 69: 2110 (1972).

C.2 Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source, or intended host.

Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1 μg plasmid or ·DNA fragments is used with about 1 unit of enzyme in about 20 μl of buffer solution. (Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer.) Incubation times of about 1 hour at 37° C. are workable. After incubations, protein is removed by extraction with phenol and chloroform, and the nucleic acid is recovered from the aqueous fraction by precipitation with ethanol.

If blunt ends are required, the preparation is treated for 15 minutes at 15° with 10 units of *E. coli* DNA Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments is performed using 6 percent polyacrylamide gel described by Goeddel, D., et al, *Nucleic Acids Res.*, 8: 4057 (1980) incorporated herein by reference.

For ligation, approximately equimolor amounts of the desired components, suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5 μg DNA. (When cleaved vectors are used as components, it may be useful to prevent religation of the cleaved vector by pretreatment with bacterial alkaline phosphatase.)

In the examples described below correct ligations for plasmid construction are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with the ligation mixture. Successful transformants were selected by ampicillin or tetracycline resistance depending on the mode of plasmid construction. Plasmids from the transformants were then prepared, analyzed by restriction and/or sequenced by the method of Messing, et al, *Nucleic Acids Res.*, 9: 309 (1981) or by the method of Maxam, et al, *Methods in Enzymology*, 65: 499 (1980).

D. Outline of Procedures

D.1 Mammalian Antibodies

The first type of antibody which forms a part of this invention, and is prepared by the methods thereof, is "mammalian antibody"-one wherein the heavy and light chains mimic the amino acid sequences of an antibody otherwise produced by a mature mammalian B lymphocyte either in situ or when fused with an immortalized cell as part of a hybridoma culture. In outline, these antibodies are produced as follows:

Messenger RNA coding for heavy or light chain is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT cellulose chromatography to segregate the poly-A mRNA.. The poly-A mRNA may, further, be fractionated to obtain sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as the case may be.

A cDNA library is then prepared from the mixture of mRNA using a suitable primer, preferably a nucleic

acid sequence which is characteristic of the desired cDNA. Such a primer may be hypothesized and synthesized based on the amino acid sequence of the antibody if the sequence is known. In the alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *E. coli*, is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, tetracycline resistance or other phenotypic characteristic residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with ATP^{32}. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

The tailored gene sequence is then positioned in a vector which contains a promoter in reading frame with the gene and compatible with the proposed host cell. A number of plasmids such as those described in U.S. Pat. Nos. 4,663,283 and 4,456,748 and pending U.S. Ser. No. 291,892, have been described which already contain the appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, as well as convenient markers.

In the present invention, the gene coding for the light chain and that coding for the heavy chain are recovered separately by the procedures outlined above. Thus they may be inserted into separate expression plasmids, or together in the same plasmid, so long as each is under suitable promoter and translation control.

The expression vectors constructed above are then used to transform suitable cells. The light and heavy chains may be transformed into separate cell cultures, either of the same or of differing species; separate plasmids for light and heavy chain may be used to co-transform a single cell culture, or, finally, a single expression plasmid containing both genes and capable of expressing the genes for both light and heavy chain may be transformed into a single cell culture.

Regardless of which of the three foregoing options is chosen, the cells are grown under conditions appropriate to the production of the desired protein. Such condi-

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tions are primarily mandated by the type of promoter and control systems used in the expression vector, rather than by the nature of the desired protein. The protein thus produced is then recovered from the cell culture by methods known in the art, but choice of which is necessarily dependent on the form in which the protein is expressed. For example, it is common for mature heterologous proteins expressed in *E. coli* to be deposited within the cells as insoluble particles which require cell lysis and solubilization in denaturant to permit recovery. On the other hand, proteins under proper synthesis circumstances, in yeast and bacterial strains, can be secreted into the medium (yeast and gram positive bacteria) or into the periplasmic space (gram negative bacteria) allowing recovery by less drastic procedures. Tissue culture cells as hosts also appear, in general, to permit reasonably facile recovery of heterologous proteins.

When heavy and light chain are coexpressed in the same host, the isolation procedure is designed so as to recover reconstituted antibody. This can be accomplished in vitro as described below, or might be possible in vivo in a microorganism which secretes the IgG chains out of the reducing environment of the cytoplasm. A more detailed description is given in D.2, below.

D.2 Chain Recombination Techniques

The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique and unprecedented assemblies of immunoglobulins, Fab regions, and univalent antibodies. Such preparations require the use of techniques to reassemble isolated chains. Such means are known in the art, and it is, thus, appropriate to review them here.

While single chain disulfide bond containing proteins have been reduced and reoxidized to regenerate in high yield native structure and activity (Freedman, R. B., et al. In *Enzymology of Post Translational Modification of Proteins*, I: 157-212 (1980) Academic Press, NY.), proteins which consist of discontinuous polypeptide chains held together by disulfide bonds are more difficult to reconstruct in vitro after reductive cleavage. Insulin, a cameo case, has received much experimental attention over the years, and can now be reconstructed so efficiently that an industrial process has been built around it (Chance, R. E., et al., In *Peptides: Proceedings of the Seventh Annual American Peptide Symposium* (Rich, D. H. and Gross, E., eds.) 721-728, Pierce Chemical Co., Rockford, IL. (1981)).

Immunoglobulin has proved a more difficult problem than insulin. The tetramer is stabilized intra and intermolecularly by 15 or more disulfide bonds. It has been possible to recombine heavy and light chains, disrupted by cleavage of only the interchain disulfides, to regain antibody activity even without restoration of the interchain disulfides (Edelman, G. M., et al., *Proc. Natl. Acad. Sci (USA)* 50: 753 (1963)). In addition, active fragments of IgG formed by proteolysis (Fab fragments of ~50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give active antibody (Haber, E., *Proc. Natl. Acad. Sci. (USA)* 52: 1099 (1964); Whitney, P. L., et al., *Proc. Natl. Acad. Sci. (USA)* 53: 524 (1965)). Attempts to reconstitute active antibody from fully reduced native IgG have been largely unsuccessful, presumably due to insolubility of the reduced chains and of side products or intermediates in the refolding

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pathway (see discussion in Freedman, M. H., et al., *J. Biol. Chem.* 241: 5225 (1966)). If, however, the immunoglobulin is randomly modified by polyalanylation of its lysines before complete reduction, the separated chains have the ability to recover antigen-combining activity upon reoxidation (ibid).

A particularly suitable method for immunoglobulin reconstitution is derivable from the now classical insulin recombination studies, wherein starting material was prepared by oxidative sulfitolysis, thus generating thiol-labile S-sulfonate groups at all cysteines in the protein, non-reductively breaking disulfides (Chance et al. (supra)). Oxidative sulfitolysis is a mild disulfide cleavage reaction (Means, G. E., et al., *Chemical Modification of Proteins*, Holden-Day, San Francisco (1971) which is sometimes more gentle than reduction (Wetzel, R., *Biochemistry*, submitted (1983)), and which generates derivatives which are stable until exposed to mild reducing agent at which time disulfide reformation can occur via thiol-disulfide interchange (Morehead, H., et al. *Biochemistry*, in press, (1983)). In the present invention the heavy and light chain S-sulfonates generated by oxidative sulfitolysis were reconstituted utilizing both air oxidation and thiol-disulfide interchange to drive disulfide bond formation. The general procedure is set forth in detail in U.S. Pat. No. 4,599,197 incorporated herein by reference.

D.3 Variants Permitted by Recombinant Technology

Using the techniques described in paragraphs D.1 and D.2, additional operations which were utilized to gain efficient production of mammalian antibody can be varied in quite straightforward and simple ways to produce a great variety of modifications of this basic antibody form. These variations are inherent in the use of recombinant technology, which permits modification at a genetic level of amino acid sequences in normally encountered mammalian immunoglobulin chains, and the great power of this approach lies in its ability to achieve these variations, as well as in its potential for economic and specific production of desired scarce, and often contaminated, molecules. The variations also inhere in the ability to isolate production of individual chains, and thus create novel assemblies.

Briefly, since genetic manipulations permit reconstruction of genomic material in the process of construction of expression vectors, such reconstruction can be manipulated to produce new coding sequences for the components of "natural" antibodies or immunoglobulins. As discussed in further detail below, the coding sequence for a mammalian heavy chain may not be derived entirely from a single source or a single species, but portions of a sequence can be recovered by the techniques described in D.1 from differing pools of mRNA, such as murine-murine hybridomas, human-murine hybridomas, or B cells differentiated in response to a series of antigen challenges. The desired portions of the sequences in each case can be recovered using the probe and analysis techniques described in D.1, and recombined in an expression vector using the same ligation procedures as would be employed for portions of the same model sequence. Such chimeric chains can be constructed of any desired length; hence, for example, a complete heavy chain can be constructed, or only sequence for the Fab region thereof.

The additional area of flexibility which arises from the use of recombinant techniques results from the power to produce heavy and light chains or fragments thereof in separate cultures or of unique combinations

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of heavy and light chain in the same culture, and to prevent reconstitution of the antibody or immunoglobulin aggregation until the suitable components are assembled. Thus, while normal antibody production results automatically in the formation of "mammalian antibodies" because the light and heavy chain portions are constructed in response to a particular determinant in the same cell, the methods of the present invention present the opportunity to assemble entirely new mixtures. Somewhat limited quantities of "hybrid" antibodies have been produced by "quadromas" i.e., fusions of two hybridoma cell cultures which permit random assemblies of the heavy and light chains so produced.

The present invention permits a more controlled assembly of desired chains, either by mixing the desired chains in vitro, or by transforming the same culture with the coding sequences for the desired chains.

D.4 Composite Immunoglobulins

The foregoing procedure, which describes in detail the recombinant production of mammalian antibodies is employed with some modifications to construct the remaining types of antibodies or NSIs encompassed by the present invention. To prepare the particular embodiment of composite non-specific immunoglobulin wherein the homology of the chains corresponds to the sequences of immunoglobulins of different specificities, it is of course, only necessary to prepare the heavy and light chains in separate cultures and reassemble them as desired.

For example, in order to make an anti-CEA light chain/anti-hepatitis heavy chain composite antibody, a suitable source for the mRNA used as a template for the light chain clone would comprise, for instance, the anti CEA producing cell line of paragraph E.1. The mRNA corresponding to heavy chain would be derived from B cells raised in response to hepatitis infection or from hybridoma in which the B cell was of this origin. It is clear that such composites can be assembled using the methods of the invention almost at will, and are limited only by available sources of mRNA suitable for use as templates for the respective chains. All other features of the process are similar to those described above.

D.5 Hybrid Antibodies

Hybrid antibodies are particularly useful as they are capable of simultaneous reaction with more than one antigen. Pairs of heavy and light chains corresponding to chains of antibodies for different antigens, such as those set forth in paragraph D.4 are prepared in four separate cultures, thus preventing premature assembly of the tetramer. Subsequent mixing of the four separately prepared peptides then permits assembly into the desired tetramers. While random aggregation may lead to the formation of considerable undesired product, that portion of the product in which homologous light and heavy chains are bound to each other and mismatched to another pair gives the desired hybrid antibody.

D.6 Chimeric Antibodies

For construction of chimeric antibodies (wherein, for example, the variable sequences are separately derived from the constant sequences) the procedures of paragraph D.1 and D.2 are again applicable with appropriate additions and modifications. A preferred procedure is to recover desired portions of the genes encoding for parts of the heavy and light chains from suitable, differing, sources and then to religate these fragments using restriction endonucleases to reconstruct the gene coding for each chain.

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For example, in a particular preferred chimeric construction, portions of the heavy chain gene and of the light chain gene which encode the variable sequences of antibodies produced by a murine hybridoma culture are recovered and cloned from this culture and gene fragments encoding the constant regions of the heavy and light chains for human antibodies recovered and cloned from, for example, human myeloma cells. Suitable restriction enzymes may then be used to ligate the variable portions of the mouse gene to the constant regions of the human gene for each of the two chains. The chimeric chains are produced as set forth in D.1, aggregated as set forth in D.2 and used in the same manner as the non-chimeric forms. Of course, any splice point in the chains can be chosen.

D.7 Altered Antibodies

Altered antibodies present, in essence, an extension of chimeric ones. Again, the techniques of D.1 and D.2 are applicable; however, rather than splicing portions of the chain(s), suitable amino acid alterations, deletions or additions are made using available techniques such as mutagenesis (supra). For example, genes which encode antibodies having diminished complement fixation properties, or which have enhanced metal binding capacities are prepared using such techniques. The latter type may, for example, take advantage of the known gene sequence encoding metalothionein II (Karin, M., et al., *Nature*, 299: 797 (1982)). The chelating properties of this molecular fragment are useful in carrying heavy metals to tumor sites as an aid in tumor imaging (Scheinberg, D. A., et al., *Science*, 215: 19 (1982).

D.8 Univalent Antibodies

In another preferred embodiment, antibodies are formed which comprise one heavy and light chain pair coupled with the Fc region of a third (heavy) chain. These antibodies have a particularly useful property. They can, like ordinary antibodies, be used to target antigenic surfaces of tissues, such as tumors, but, unlike ordinary antibodies, they do not cause the antigenic surfaces of the target tissue to retreat and become non-receptive. Ordinary antibody use results in aggregation and subsequent inactivation, for several hours, of such surface antigens.

The method of construction of univalent antibodies is a straightforward application of the invention. The gene for heavy chain of the desired Fc region is cleaved by restriction enzymes, and only that portion coding for the desired Fc region expressed. This portion is then bound using the technique of D.2 to separately produced heavy chain the desired pairs separated from heavy/heavy and Fc/Fc combinations, and separately produced light chain added. Pre-binding of the two heavy chain portions thus diminishes the probability of formaion of ordinary antibody.

D.9 Fab Protein

Similarly, it is not necessary to include the entire gene for the heavy chain portion. All of the aforementioned variations can be superimposed on a procedure for Fab protein production and the overall procedure differs only in that that portion of the heavy chain coding for the amino terminal 220 amino acids is employed in the appropriate expression vector.

E. Specific Examples of Preferred Embodiments

The invention has been described above in general terms and there follow several specific examples of embodiments which set forth details of experimental procedure in producing the desired antibodies. Example E.1 sets forth the general procedure for preparing anti

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CEA antibody components, i.e. for a "mammalian antibody". Example E.3 sets forth the procedure for reconstitution and thus is applicable to preparation of mammalian, composite, hybrid and chimeric immunoglobulins, and Fab proteins and univalent antibodies. Example E.4 sets forth the procedure for tailoring the heavy or light chain so that the variable and constant regions may be derived from different sources. Example E.5 sets forth the method of obtaining a shortened heavy chain genome which permits the production of the Fab regions and, in an analogous manner, Fc region.

The examples set forth below are included for illustrative purposes and do not limit the scope of the invention.

E.1 Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis

Carcinoembryonic antigen (CEA) is associated with the surface of certain tumor cells of human origin (Gold, P., et al., *J. Exp. Med.*, 122: 467 (1965)). Antibodies which bind to CEA (anti-CEA antibodies) are useful in early detection of these tumors (Van Nagell, T. R., et al., *Cancer Res.* 40: 502 (1980)), and have the potential for use in treatment of those human tumors which appear to support CEA at their surfaces. A mouse hybridoma cell line which secretes anti-CEA antibodies of the Igγ₁ class, CEA.66-E3, has been prepared as described by Wagener, C, et al., *J. Immunol.* (in press) which is incorporated herein by reference, and was used as mRNA source. The production of anti CEA antibodies by this cell line was determined. The N-terminal sequences of the antibodies produced by these cells was compared with those of monoclonal anti CEA as follows. Purified IgG was treated with PCAse (Podell, D. N., et al., BBRC 81: 176 (1978)), and then dissociated in 6M guanidine hydrochloride, 10 mM 2-mercaptoethanol (1.0 mg of immunoglobulin, 5 min, 100° C. water bath). The dissociated chains were separated on a Waters Associates alkyl phenyl column using a linear gradient from 100 percent A (0.1 percent TFA-water) to 90 percent B (TFA/H₂O/MeCN 0.1/9.9/90) at a flow rate of 0.8 ml/min. Three major peaks were eluted and analyzed on SDS gels by silver staining. The first two peaks were pure light chain (MW 25,000 daltons), the third peak showed a (7:3) mixture of heavy and light chain. 1.2 nmoles of light chain were sequenced by the method of Shively, J. E., *Methods in Enzymology*, 79: 31 (1981), with an NH₂-terminal yield of 0.4 nmoles. A mixture of heavy and light chains (3 nmoles) was also sequenced, and sequence of light chain was deducted from the double sequence to yield the sequence of the heavy chain.

In the description which follows, isolation and expression of the genes for the heavy and light chains for anti CEA antibody produced by CEA.66-E3 are described. As the constant regions of these chains belong to the gamma and kappa families, respectively, "light chain" and "kappa chain", and "heavy chain" and "gamma chain", respectively, are used interchangeably below.

E.1.1 Isolation of Messenger RNA for Anti CEA Light and Heavy (Kappa and Gamma) Chains

Total RNA for CEA.66-E3 cells was extracted essentially as reported by Lynch et al, *Virology*, 98: 251 (1979). Cells were pelleted by centrifugation and approximately 1 g portions of pellet resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM MgCl₂. The resuspended cells were lysed by addition of non-ionic detergent NP-40 to a final concentration of 1

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percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant was extracted twice with 3 ml portions of phenol (redistilled)/chloroform:isoamyl alcohol 25:1 at 4° C. The aqueous phase was made 0.2M in NaCl and total RNA was precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA was purified from total RNA by oligo-dT cellulose chromatography as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci (USA)*, 69: 1408 (1972). 142 μg of polyA mRNA was obtained from 1 g cells.

E.1.2 Preparation of *E. coli* Colony Library Containing Plasmids with Heavy and Light DNA Sequence Inserts

5 μg of the unfractionated polyA mRNA prepared in paragraph E.1.1 was used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures as described by Goeddel et al., *Nature* 281: 544 (1979) and Wickens et al., *J. Biol. Chem.* 253: 2483 (1978) incorporated herein by reference. The cDNA was size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length was recovered by electroelution. A 20 ng portion of ds cDNA was extended with deoxy C residues using terminal deoxynucleotidyl transferase as described in Chang et al., *Nature* 275: 617 (1978) incorporated herein by reference, and annealed with 200 ng of the plasmid pBR322 (Bolivar et al., *Gene* 2: 95 (1977)) which had been cleaved with Pst I and tailed with deoxy G. Each annealed mixture was then transformed into *E. coli* K12 strain 294 (ATCC No 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants were obtained.

E.1.3 Preparation of Synthetic Probes

The 14 mer, 5'GGTGGGAAGATGGA 3' complementary to the coding sequence of constant region for mous MOPC21 kappa chain which begins 25 basepairs 3' of the variable region DNA sequence was used as kappa chain probe. A 15 mer, 5'GACCAGGCATC-CCAG 3', complementary to a coding sequence located 72 basepairs 3' of the variable region DNA sequence for mouse MOPC21 gamma chain was used to probe gamma chain gene.

Both probes were synthesized by the phosphotriester method described in German Offenlegungschrift No 2644432, incorporated herein by reference, and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide were combined in 25 μl of 60 mM Tris HCl (pH 8), 10 mM MgCl₂, 15 mM beta-mercaptoethanol, and 100 μCi (γ-³²P) ATP (Amersham, 5000 Ci/mMole). 5 units of T4 polynucleotide kinase were added and the reaction was allowed to proceed at 37° C. for 30 minutes and terminated by addition of EDTA to 20 mM.

E.1.4 Screening of Colony Library for Kappa or Gamma Chain Sequences

~2000 colonies prepared as described in paragraph E.1.2 were individually inoculated into wells of microtitre dishes containing LB (Miller, Experiments in Molecular Genetics, p. 431-3, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. (1972))+5 μg/ml tetracycline and stored at −20° C. after addition of DMSO to 7 percent. Individual colonies from this library were transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 μg/ml tetracycline. After ~10 hours growth at 37° C. the colony filters

were transferred to agar plates containing LB+5 μg/ml tetracycline and 12.5 μg/ml chloramphicol and reincubated overnight at 37° C. The DNA from each colony was then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure as described in Grunstein et al., *Proc. Natl. Acad. Sci. (USA)* 72: 3961 (1975), incorporated herein by reference. Each filter was floated for 3 minutes on 0.5N NaOH, 1.5M NaCl to lyse the colonies and denature the DNA then neutralized by floating for 15 minutes on 3M NaCl, 0.5M Tris HCl (pH 7.5). The filters were then floated for an additional 15 minutes on 2×SSC, and subsequently baked for 2 hours in an 80° C. vacuum oven. The filters were prehybridized for ~2 hours at room temperature in 0.9M NaCl, 1× Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 μg/ml *E. coli* t-RNA, and hybridized in the same solution overnight, essentially as described by Wallace et al. *Nucleic Acids Research* 9: 879 (1981) using ~40×10⁶ cpm of either the kinased kappa or gamma probe described above.

After extensive washing at 37° C. in 6X SSC, 0.1 percent SDS, the filters were exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens from 16–24 hours at −80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe were characterized.

E.1.5 Characterization of Colonies which Hybridize to Kappa DNA Sequence Probe

Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe were cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain. The complete nucleotide sequence of the cDNA insert of one of these plasmids was determined by the dideoxynucleotide chain termination method as described by Smith, *Methods Enzymol.* 65, 560 (1980) incorporated herein by reference after subcloning restriction endonuclease cleavage fragments into M13 vectors (Messing et al., *Nucleic Acids Research* 9: 309 (1981). FIG. 2 shows the nucleotide sequence of the cDNA insert of pK17G4 and FIG. 3 shows the gene sequence with the corresponding amino acid sequence. Thus, the entire coding region of mouse anti-CEA kappa chain was isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acid 215 begins 212 basepairs of 3' untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides 374–388 (FIG. 2).

E.1.6. Characterization of Colonies which Hybridize to Gamma 1 DNA Probe

Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe was subjected to Pst I restriction endonuclease analysis as described in E.1.5. Plasmid DNAs demonstrating the largest cDNA insert fragments were selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region. Selected plasmid DNAs were digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

In one plasmid isolated, p γ298 the cDNA insert of about 1300 bp contains sequence information for the 5' untranslated region, the signal sequence and the N-terminal portion of heavy chain. Because pγ298 did not encode the C-terminal sequence for mouse anti-CEA gamma 1 chain, plasmid DNA was isolated from other colonies and screened with PstI and NcoI. The C-terminal region of the cDNA insert of pγ11 was sequenced and shown to contain the stop codon, 3' untranslated sequence and that portion of the coding sequence missing from p γ298.

FIG. 4 presents the entire nucleotide sequence of mouse anti-CEA heavy chain (as determined by the dideoxynucleotide chain termination method of Smith, *Methods Enzymol.* 65: 560 (1980)) and FIG. 5 includes the translated sequence.

The amino acid sequence of gamma 1 (heavy chain) deduced from the nucleotide sequence of the pγ298 cDNA insert corresponds perfectly to the first 23 N-terminal amino acids of mature mouse anti-CEA gamma 1 chain as determined by amino acid sequence analysis of purified mouse anti-CEA gamma-1 chain. The coding region consists of 57 basepairs or 19 amino acids of presequences and 1346 basepairs or 447 amino acids of mature protein. The mature unglycosolated protein (MW 52,258) has a variable region of 135 amino acids, including a D region of 12 amino acids, and a J4 joining region of 13 amino acids. The constant region is 324 amino acids. After the stop codon behind amino acid 447 begins 96 bp of 3' untranslated sequences up to the polyA addition. The probe used to identify pγ298 and pγ11 hybridized to nucleotides 528–542 (FIG. 4).

E.1.7 Construction of a Plasmid For Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene. pKCEAtrp207-1*

FIG. 6 illustrates the construction of pKCEAtrp207-1*

First, an intermediate plasmid pHGH207-1*, having a single trp promoter, was prepared as follows:

The plasmid pHGH 207 (described in U.S. Pat. No. 4,663,283), was used to prepare pHGH 207-1. pHGH 207 was digested with BamHI, followed by partial digestion with EcoRI. The largest fragment, which contains the entire trp promoter, was isolated and ligated to the largest EcoRI-BamHI fragment from pBR322, and the ligation mixture used to transform *E. coli* 294. Tet and Amp resistant colonies were isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoRI site between the ampᴿ gene and the trp promoter, was obtained by partial digestion of pHGH207-1 (de Boer et al., *Promoters.* Rodriquez et al. Eds. (1982), pp. 462–481 U.S. Pat. No. 4,663,283) with

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EcoR I, filling in the ends with Klenow and dNTPs, and religation.

5 μg of pHGH207-1* was digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 μl reaction containing 60 mM NaCl, 7 mM MgCl₂, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C. for 1 hour, followed; by extraction with phenol/CHCl₃ and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/CHCl₃ extraction and ethanol precipitation.

The DNA was resuspended in 50 μl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30′ at 65° followed by phenol/CHCl₃ extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence was prepared as follows: 7 μg of pK17G4 DNA was digested with Pst I and the kappa chain containing cDNA insert was isolted by 6 percent gel electrophoresis, and electroelution. After phenol/CHCl₃ extraction, ethanol precipitaion and resuspensionin water, this fragment was digested with Ava II. The 333 bp Pst I-Ava II DNA fragment was isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer was synthesized by the phosphotriester method G.O. 2,644,432 (supra) and has the following sequence:

Met Asp Ile Val Met
5′ ATG GAC ATT GTT ATG 3′

The 5′ methionine serves as the initiation codon. 500 ng of this primer was phosphorylated at the 5′ end with 10 units T4 DNA kinase in 20 μl reaction containing 0.5 mM ATP. ~200 ng of the Pst I-Ava II DNA fragment was mixed with the 20 μl of the phosphorylated primer, heated to 95° C. for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution was made 60 mM NaCl, 7 mM MgCl₂, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C. this primer repair reaction was phenol/CHCl₃ extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture was then electrophoresed on a 6 percent polyacrylamide gel and ~50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) was obtained after electroelution.

100 ng of fragment 1 (supra) and 50 ng of fragment 2 were combined in 20 μl of 20 mM Tris HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C. the reaction was transformed into E. coli K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequene analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1 (FIG. 6).

The remainder of the coding sequence of the kappa light chain gene was prepared as follows:

The Pst I cDNA insert fragment from 7 μg of K17G4 DNA was partially digested with Ava II and the Ava II cohesive ends were extended to blunt ends in a DNA Polymerase I large fragment reaction. Following 6 percent polyacrylamide gel electrophoresis the 686

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basepair Pst I to blunt ended Ava II DNA fragment was isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) was isoated and purified after gel electrophoresis.

10 μg of pKCEAInt1 DNA was digested with Ava I, extended with DNA polymerase I large fragment. and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment were isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) was treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment was digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) was purified. ~75 ng of fragment 4, ~50 ng of fragment 3 and ~50 ng of fragment 5 were combined in a T4 DNA ligase reactin and incubated overnight at 14°, and the reaction mixture transformed into E. coli K12 strain 294. Plasmid DNA from six ampicillin resistant transformants were analyzed by restriction endonuclease digestion. One plasmid DNA demonstrated the proper construction and was digested pKCEAInt2.

Final construction was effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared by deletion of the region of pBR322 between its unique AvaI and PvaII sites)

The K-CEA fragment was prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) was prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I. followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments as prepared in the preceding paragraphs were ligated with T4 DNA ligase, and the ligation mixture transformed into E. coli as above. Plasmid DNA from several ampicillin resistant transformants were selected for analysis. and one plasmid DNA demonstrated the proper construction. and was designated pKCEAtrp207-1*.

E.1.8 Construction of a Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1*

FIG. 7 illustrates the construction of pγCEAtrp207-1*. This plasmid was constructed in two parts beginning with construction of the C-terminal regin of the gamma 1 gene.

5 μg of plasmid pHGH207-1* was digested with Ava I, extended to blunt ends with DNA polymerase I large fragment (Klenow fragment), extracted with phenol/CHCl₃, and ethanol precipitated. The DNA was digested with BamH I treated with BAP and the large fragment (fragment A) was purified by 6 percent polyacrylamide gel electrophoresis and electroelution.

~5 μg of pγ11 was digested with Pst I and the gamma chain cDNA insert fragment containing the C-terminal portion of the gene was purified. digested with Ava II followed by extension of the Ava II cohesive ends with Klenow, followed by Taq I digestion. The 375 basepair blunt ended Ava II to Taq I fragment

(fragment B) was isolated and purified by gel electrophoresis and electroelution.

9 μg of py298 was digested with Taq I and BamH I for isolation of the 496 basepair fragment (fragment C).

Approximately equimolar amounts of fragments A, B, and C were ligated overnight at 14° in 20 μl reaction mixture, then transformed into *E. coli* strain 294. The plasmid DNA from six ampicillin resistant transformants was committed to restriction endonuclease analysis and one plasmid DNA, named pyCEAInt, demonstrated the correct construction of the C-terminal portion of gamma 1 (FIG. 5).

To obtain the N-terminal sequences, 30 μg of py298 was digested with Pst I and the 628 basepair DNA fragment encoding the N-terminal region of mouse anti-CEA gamma chain was isolated and purified. This fragment was further digested with Alu I and Rsa I for isolation of the 280 basepair fragment. A 15 nucleotide DNA primer

met glu val met leu
5' ATG GAA GTG ATG CTG 3'

was synthesized by the phosphotriester method (supra).

The 5' methionine serves as the initiation codon. 500 ng of this synthetic oligomer primer was phosphorylated at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl reaction mixture. ~500 ng of the 280 basepair Alu I-Rsa I DNA fragment was mixed with the phosphorylated primer. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM MgCl₂, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2 hours incubation at 37° C., this primer repair reaction was phenol/CHCl₃ extracted, ethanol precipitated, and digested to completion with HpaII. ~50 ng of the expected 125 basepair blund-end to Hpa II DNA fragment (fragment D) was purified from the gel.

A second aliquot of py298 DNA was digested with Pst I, the 628 basepair DNA fragment purified by polyacrylamide gel electrophoresis, and further digested with BamH I ahd Hpa II. The resulting 380 basepair fragment (fragment E) was purified by gel electrophoresis.

~5 μg of pyCEAIntI was digested with EcoR I, the cohesive ends were made flush with DNA polymerase I (Klenow), further digested with BamH I, treated with BAP and electrophoresed on a 6 percent polyacrylamide gel. The large vector fragment (fragment F) was isolated and purified.

In a three fragment ligation, 50 ng fragment D, 100 ng fragment E, and 100 ng fragment F were ligated overnight at 4° in a 20 μreaction mixture and used to transform *E. coli* K12 strain 294. The plasmid DNAs from 12 ampicillin resistant transformants were analyzed for the correct construction and the nucleotide sequence surrounding the initiation codon was verified to be correct for the plasmid named pyCEAInt2.

The expression plasmid, pyCEAtrp207-1* used for expression of the heavy chain gene is prepared by a 3-way ligation using the large vector fragment from pBR322(XAP) (supra) and two fragments prepared from pyCEAInt2.

pBR322(XAP) was treated as above by digestion with EcoR1, blunt ending with DNA polymerase (Klenow) in the presence of dNTPs, followed by digestion

with Pst I, and isolation of the large vector fragment by gel electrophoresis. A 1543 base pair fragment from pyCEAInt2 containing trp promoter linked with the N-terminal coding region of the heavy chain gene was isolated by treating pyCEAInt2 with Pst I followed by BamH I, and isolation of the desired fragment using PAGE. The 869 base pair fragment containing the C-terminal coding portion of the gene was prepared by partial digestion of pyCEAInt2 with Ava I, blunt ending with Klenow, and subsequent digestion with BamH I, followed by purification of the desired fragment by gel electrophoresis.

The aforementioned three fragments were then ligated under standard conditions using T4 DNA ligase, and a ligation mixture used to transform *E. coli* strain 294. Plasmid DNAs from several tetracycline resistant transformants were analyzed; one plasmid DNA demonstrated the proper construction and was designated pyCEAtrp207-1*.

E.1.9. Production of Immunoglobulin Chains by *E. coli*

E. coli strain W3110 (ATTC No. 27325) was transformed with pyCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

To obtain double transformants, *E. coli* strain W3110 cells were transformed with a modified pKCEAtrp207-1*, pKCEAtrp207-1*Δ, which had been modified by cleaving a Pst I-Pvu I fragment from the amp^R gene and religating. Cells transformed with pKCEAtrp207-1*Δ are thus sensitive to ampicillin but still resistant to tetracycline. Successful transformants were retransformed using pyCEAInt2 which confers resistance to ampicillin but not tetracycline. Cells containing both pKCEAtrp207-1*Δ and pyCEAInt2 thus identified by growth in a medium containing both ampicillin and tetracycline.

To confirm the production of heavy and/or light chains in the transformed cells, the cell samples were inoculated into M9 tryptophan free medium containing 10 μg/ml tetracycline, and induced with indoleacrylic acid (IAA) when the OD 550 reads 0.5. The induced cells were grown at 37° C. during various time periods and then spun down, and suspended in TE buffer containing 2 percent SDS and 0.1M β-mercaptoethanol and boiled for 5 minutes. A 10×volume of acetone was added and the cells kept at 22° C. for 10 minutes, then centrifuged at 12,000 rpm. The precipitate was suspended in O'Farrell SDS sample buffer (O'Farrell. P. H., *J. Biol. Chem.*, 250: 4007 (1975)); boiled 3 minutes, recentrifuged, and fractionated using SDS PAGE (10 percent), and stained with silver stain (Goldman. D. et al., *Science* 211: 1437 (1981)); or subjected to Western blot using rabbit anti-mouse IgG (Burnett, W. N., et al., *Anal. Biochem.* 112: 195 (1981)), for identification light chain and heavy chain.

Cells transformed with pyCEAtrp207-1* showed bands upon SDS PAGE corresponding to heavy chain molecular weight as developed by silver stain. Cells transformed with pKCEAtrp207-1* showed the proper molecular weight band for light chain as identified by Western blot; double transformed cells showed bands for both heavy and light chain molecular weight proteins when developed using rabbit anti-mouse IgG by Western blot. These results are shown in FIGS. 8A, 8B, and 8C.

FIG. 8A shows results developed by silver stain from cells transformed with pyCEAtrp207-1*. Lane 1 is

monoclonal anti-CEA heavy chain (standard) from CEA.66-E3. Lanes 2b-5b are timed samples 2 hrs, 4 hrs, 6 hrs, and 24 hrs after IAA addition. Lanes 2a-5a are corresponding untransformed controls; Lanes 2c-5c are corresponding uninduced transformants.

FIG. 8B shows results developed by Western blot from cells transformed with pKCEAtrp207-1*. Lanes 1b-6b are extracts from induced cells immediately, 1 hr, 3.5 hrs, 5 hrs, 8 hrs, and 24 hrs after IAA addition, and 1a-6a corresponding uninduced controls. Lane 7 is an extract from a pyCEAtrp207-1* control, lanes 8, 9, and 10 are varying amounts of anti CEA-kappa chain from CEA.66-E3 cells.

FIG. 8C shows results developed by Western blot from four colonies of double tranformed cells 24 hours after IAA addition (lanes 4–7). Lanes 1–3 are varying amounts of monoclonal gamma chain controls, lanes 8 and 9 are untransformed and pyCEAtrp207-1* transformed cell extracts, respectively.

In another quantitative assay, frozen, transformed E. coli cells grown according to E.1.10 (below) were lysed by heating in sodium dodecyl sulfate (SDS)/β-mercaptoethanol cell lysis buffer at 100°. Aliquots were loaded on an SDS polyacrylamide gel next to lanes loaded with various amounts of hybridoma anti-CEA. The gel was developed by the Western blot, Burnett (supra), using ^{125}I-labeled sheep anti-mouse IgG antibody from New England Nuclear. The results are shown in FIG. 9. The figure shows that the E. coli products co-migrate with the authentic hybridoma chains, indicating no detectable proteolytic degradation in E. coli. Heavy chain from mammalian cells is expected to be slightly heavier than E. coli material due to glycosylation in the former. Using the hybridoma lanes as a standard, the following estimates of heavy and light chain production were made:

	(Per gram of cells)
E. coli (W3110/pyCEAtrp207-1*)	5 mg γ
E. coli (W3110/pKCEAtrp207-1*)	1.5 mg K
E. coli (W3110/pKCEAtrp207-1*Δ, pyCEA Int2)	0.5 mg K, 1.0 mg γ

E.1.10 Reconstitution of Antibody from Recombinant K and Gamma Chains

In order to obtain heavy and light chain preparation for reconstitution, transformed cells were grown in larger batches, harvested and frozen. Conditions of growth of the variously transformed cells were as follows:

E. coli (W3110/pyCEAtrp207-1*) were inoculated into 500 ml LB medium containing 5 μg/ml tetracycline and grown on a rotary shaker for 8 hours. The culture was then transformed to 10 liters of fermentation medium containing yeast nutrients, salts, glucose, and 2 μg/ml tetracycline. Additional glucose was added during growth and at OD 550=20, indoleacrylic (IAA), a trp derepressor, was added to a concentration of 50 μg/ml. The cells were fed additional glucose to a final OD 550=40, achieved approximately 6 hours from the IAA addition.

E. coli (W3110) cells transformed with pKCEA trp 207-1* and double transformed (with pKCEAtrp207-1*Δ and pyCEAInt2) were grown in a manner analogous to that described above except that the OD 550 six hours after IAA addition at harvest was 25-30.

The cells were then harvested by centrifugation, and frozen.

E.2 Assay Method for Reconstituted Antibody

Anti-CEA activity was determined by ELISA as a criterion for successful reconstruction. Wells of microtiter plates (Dynatech Immulon) were saturated with CEA by incubation 100 μl of 2-5 μg CEA/ml solution in 0.1M carbonate buffer, pH 9.3 for 12 hours at room temperature. The wells were then washed 4 times with phosphate buffered saline (PBS), and then saturated with BSA by incubating 200 μl of 0.5 percent BSA in PBS for 2 hours at 37° C., followed by washing 4 times with PBS. Fifty microliters of each sample was applied to each well. A standard curve (shown in FIG. 10), was run, which consisted of 50 μl samples of 10 μg, 5 μg, 1 μg, 500 ng, 100 ng, 50 ng, 10 ng, 5 ng and 1 ng antiCEA/ml in 0.5 percent BSA in PBS, plus 50 μl of 0.5 percent BSA in PBS alone as a blank. All of the samples were incubated in the plate for 90 minutes at 37° C.

The plates were then washed 4 times with PBS, and sheep anti-mouse IgG-alkaline phosphate (TAGO, Inc.) was applied to each well by adding 100 μl of enzyme concentration of 24 units/ml in 0.5 percent BSA in PBS. The solution was incubated at 37° C. for 90 minutes. The plates wer washed 4 times with PBS before adding the substrate, 100 μl of a 0.4 mg/ml solution of p-nitrophenylphosphate (Sigma) in ethanolamine buffered saline, pH 9.5. The substrate was incubated 90 minutes at 37° C. for color development.

The A_{450} of each well was read by the Microelisa Auto Reader (Dynatech) set to a threshold of 1.5, calibration of 1.0 and the 0.5 percent BSA in PBS (Blank) well set to 0.000. The A_{450} data was tabulated in RS-1 on the VAX system, and the standard curve data fitted to a four-parameter logistic model. The unknown samples' concentrations were calculated based on the A_{450} data.

E.3 Reconstruction of Recombinant Antibody and Assay

Frozen cells prepared as described in paragraph E.1.10 were thawed in cold lysis buffer [10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. The lysate was partially clarified by centrifugation for 20 mins at 3,000 rpm. The supernatant was protected from proteolytic enzymes by an additional 1 mM PmSF, and used immediately or stored frozen at −80° C.; frozen lysates were never thawed more than once.

The S-sulfonate of E. coli produced anti-CEA heavy chain (γ) was prepared as follows: Recombinant E. coli cells transformed with pyCEAtrp207-1* which contained heavy chain as insoluble bodies, were lysed and centrifuged as above; the pellet was resuspended in the same buffer, sonicated and re-centrifuged. This pellet was washed once with buffer, then suspended in 6M guanidine HCl, 0.1M Tris HCl, pH 8, 1 mM EDTA, 20 mg/ml sodium sulfite and 10 mg/ml sodium tetrathionate and allowed to react at 25° for about 16 hrs. The reaction mixture was dialyzed against 8M urea, 0.1M Tris HCl, pH 8, and stored at 4°, to give a 3 mg/ml solution of γ-SSO₃.

650 μl of cell lysate from cells of various E. coli strains producing various IgG chains, was added to 500 mg urea. To this was added β-mercaptoethanol to 20 mM, Tris-HCl, pH 8.5 to 50 mM and EDTA to 1 mM, and in some experiments, γ-SSO₃ was added to 0.1 mg/ml. After standing at 25° for 30-90 mins., the reaction mixtures were dialyzed at 4° against a buffer com-

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posed of 0.1M sodium glycinate, pH 10.8, 0.5M urea, 10 mM glycine ethyl ester, 5 mM reduced glutathione, 0.1 mM oxidized glutathione. This buffer was prepared from N₂-saturated water and the dialysis was performed in a capped Wheaton bottle. After 16–48 hours, dialysis bags were transferred to 4° phosphate buffered saline containing 1 mM PMSF and dialysis continued another 16–24 hrs. Dialysates were assayed by ELISA as described in paragraph E.2 for ability to bind CEA. The results below show the values obtained by comparison with the standard curve in x ng/ml anti-CEA. Also shown are the reconstruction efficiencies calculated from the ELISA responses, minus the background (108 ng/ml) of cells producing K chain only, and from estimates of the levels of γ and K chains in the reaction mixtures.

	ng/ml anti-CEA	Percent recombination
E. coli W3110 producing IFN-αA (control)	0	—
E. coli (W3110/pKCEAtrp207-1°)	108	—
E. coli (W3110/pKCEAtrp207-1°), plus γ-SSO₃	848	0.33
E. coli (W3110/pKCEAtrp207-1°Δ pγCEA Int2)	1580	0.76
Hybridoma anti-CEA K-SSO₃ and γ-SSO₃	540	0.40

E.4 Preparation of Chimeric Antibody

FIGS. 11 and 12 show the construction of an expression vector for a chimeric heavy (gamma) chain which comprises the murine anti CEA variable region and human γ-2 constant region.

A DNA sequence encoding the human gamma-2 heavy chain is prepared as follows: the cDNA library obtained by standard techniques from a human multiple myeloma cell line is probed with 5' GGGCACT-CGACACAA 3' to obtain the plasmid containing the cDNA insert for human gamma-2 chain (Takahashi, et al., Cell 29: 671 (1982), incorporated herein by reference), and analyzed to verify its identity with the known sequence in human gamma-2 (Ellison, J., et al., Proc. Natl. Acad. Sci. (USA), 79: 1984 (1982) incorporated herein by reference).

As shown in FIG. 11, two fragments are obtained from this cloned human gamma 2 plasmid (pγ2). The first fragment is formed by digestion with PvuII followed by digestion with Ava III, and purification of the smaller DNA fragment, which contains a portion of the constant region, using 6 percent PAGE. The second fragment is obtained by digesting the pγ2 with any restriction enzyme which cleaves in the 3' untranslated region of γ2, as deduced fromthe nucleotide sequence, filling in with Klenow and dNTPs, cleaving with Ava III, and isolating the smaller fragment using 6 percent PAGE. (The choice of a two step, two fragment composition to supply the PvuII-3' untranslated fragment provides a cleaner path to product due to the proximity of the AVAIII site to the 3 terminal end thus avoiding additional restriction sites in the gene sequence matching the 3' untranslated region site.) pγCEA207-1° is digested with EcoR 1, treated with Klenow and dNTPs to fill in the cohesive end, and digested with Pvu II, the large vector fragment containing promoter isolated by 6 percent PAGE.

The location and DNA sequence surrounding the PvuII site in the mouse gamma-1 gene are identical to

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the location and DNA sequence surrounding the PvuII site in the human gamma-2 gene.

The plasmid resulting from a three way ligation of the foregoing fragments, pChim1, contains, under the influence of trp promoter, the variable and part of the constant region of murine anti-CEA gamma 1 chain, and a portion of the gamma 2 human chain. pChim1 will, in fact, express a chimeric heavy chain when tranformed into E. coli, but one wherein the change from mouse to human does not take place at the variable to constant junction.

FIG. 12 shows modification of pChim1 to construct pChim2 so that the resulting protein from expression will contain variable region from murine anti CEA antibody and constant region from the human γ-2 chain. First, a fragment is prepared from pChim1 by treating with Nco I, blunt ending with Klenow and dNTPs, cleaving with Pvu II, and isolating the large vector fragment which is almost the complete plasmid except for short segment in the constant coding region for mouse anti CEA. A second fragment is prepared from the previously described pγ2 by treating with Pvu II, followed by treating with any restriction enzyme which cleaves in the variable region, blunt ending with Klenow and dNTPs and isolating the short fragment which comprises the junction between variable and constant regions of this chain.

Ligation of the foregoing two fragments produces an intermediate plasmid which is correct except for an extraneous DNA fragment which contains a small portion of the constant region of the murine anti CEA antigen, and a small portion of the variable region of the human gamma chain. This repair can be made by excising the Xba I to Pvu II fragment and cloning into M13 phage as described by Messing et al., Nucleic Acids Res. 9: 309 (1981), followed by in vitro site directed deletion mutagenesis as described by Adelman, et al., DNA, in press (1983) which is incorporated herein by reference. The Xba I-Pvu II fragment thus modified is ligated back into the intermediate plasmid to form pChim2, this plasmid then is capable of expressing in a suitable host a cleanly constructed murine variable/human constant chimeric heavy chain.

In an analogous fashion, but using mRNA templates for cDNA construction for human kappa rather than γ chain, the expression plasmid for chimeric light chain is prepared.

The foregoing two plasmids are then double transformed into E. coli W3110, the cells grown and the chains reconstituted as set forth in paragraph E.1-E.3 supra.

E.5 Preparation of Altered Murine Anti-CEA Antibody

E.5.1 Construction of Plasmid Vectors for Direct Expression of Altered Murine Anti-CEA Heavy Chain Gene

The cysteine residues, and the resultant disulfide bonds in the region of amino acids 216–230 in the constant region of murine anti-CEA heavy chain are suspected to be imported for complement fixation (Klein, et al., Proc. Natl. Acad. Sci. (USA), 78: 524 (1981)) but not for the antigen binding property of the resulting antibody. To decrease the probability of incorrect disulfide bond formation during reconstruction according to the process of the invention herein, the nucleotides encoding the amino acid residues 226–232 which includes codons for three cysteines, are deleted as follows:

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A "deleter" deoxyoligonucelotide, 5' CTAACAC-CATGTCAGGGT is used to delete the relevant portions of the gene from pγCEAtrp207-1* by the procedure of Wallace, et al., *Science.* 209: 1396 (1980). Briefly, the "deleter" deoxyoligonucelotide is annealed with denatured pγCEAtrp207-1* DNA, and primer repair synthesis carried out in vitro, followed by screening by hybridization of presumptive deletion clones with P32 labelled deleter sequence.

E.5.2 Production of Cysteine Deficient Altered Antibody

The plasmid prepared in E.5.1 is transformed into an *E.coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains, and the altered antibody reconstituted as described in E.1.10.

E.6 Preparation of Fab

E.6.1 Construction of a Plasmid Vector for Direct Expression of Murine Anti-CEA Gamma 1 Fab Fragment Gene pγCEAFabtrp207-1*

FIG. 13 presents the construction of pγCEA-Fabtrp207-1*. 5 μg of pBR322 was digested with Hind III, the cohesive ends made flush by treating with Klenow and dNTPs; digested with Pst I, and treated with BAP. The large vector fragment, fragment I, was recovered using 6 percent PAGE followed by electroelution.

5 μg of pγCEAtrp207-1* was digested with both BamH I and Pst I and the ~1570 bp DNA fragment (fragment II) containing the trp promoter and the gene sequence encoding the variable region continuing into constant region and further into the anti-CEA gamma 1 chain hinge region, was isolated and purified after electrophoresis.

Expression of the anti-CEA gamma 1 chain Fab fragment rather than complete heavy chain requires that a termination condon be constructed at the appropriate location in the gene. For this, the 260 bp Nco I-Nde I DNA fragment from 20 μg of the pγ298 was isolated and purified. A 13 nucleotide DNA primer, the complement of which encodes the last 3 C-terminal amino acids of the Fab gene and 2 bases of the 3 needed for the stop codon, was synthesized by the phosphotriester method (supra). The probe hybridizes to nucleotides 754 to 767 (FIG. 4) which has the following sequence:

```
       Asp Cys Gly Stop
     5' GGGATTGTGGTTG 3'
```

The third base of the stop codon is provided by the terminal nucleotide of the filled-in Hind III site from pBR322 cleavage described above. 500 ng of this primer was used in a primer repair reaction by phosphorylation at the 5' end in a reaction with 10 units T4 DNA kinase containing 0.5 mM ATP in 20 μl, and mixing with ~200 ng of the Nco I-Nde I DNA fragment. The mixture was heat denatured for 3 minutes at 95° and quenched in dry-ice ethanol. The denatured DNA solution was made 60 mM NaCl, 7 mM MgCl2, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA Polymerase I-Large Fragment was added. After 2

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hours incubation at 37° C., this primer repair reaction was phenol/CHCl3 extracted, ethanol precipitated, digested with BamH I and the reaction electrophoresed through a 6 percent polyacrylamide gel. ~50 ng of the 181 bp blunt end to BamH I DNA fragment, fragment III, was isolated and purified.

~100 ng of fragment I, ~100 ng each of fragments II and III were ligated overnight and transformed into *E. coli* K12 strain 294. Plasmid DNA from several tetracycline resistant transformants was analyzed for the proper construction and the nucleotide sequence through the repair blunt end filled-in Hind III junction was determined for verification of the TGA stop codon.

E.6.2 Production of Fab Protein

The plasmid prepared in E.6.1 is transformed into an *E. coli* strain previously transformed with pKCEAtrp207-1* as described above. The cells are grown, extracted for recombinant antibody chains and the Fab protein reconstituted as described in E.1.10.

We claim:

1. A method comprising
   (a) preparing a DNA sequence encoding a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen wherein a constant region is homologous to the corresponding constant region of an antibody of a first mammalian species and a variable region thereof is homologous to the variable region of an antibody derived from a second, different mammalian species;
   (b) inserting the sequence into a replicable expression vector operably linked to a suitable promoter compatible with a host cell;
   (c) transforming the host cell with the vector of (b);
   (d) culturing the host cell; and
   (e) recovering the chimeric heavy or light chain from the host cell culture.

2. The method of claim 1 wherein the first mammalian species is human.

3. A composition comprising a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen having a constant region homologous to a corresponding constant region of an antibody of a first mammalian species and a variable region homologous to a variable region of an antibody derived from a second, different mammalian species.

4. The chimeric heavy or light chain of claim 3 wherein the constant region is human.

5. A replicable expression vector comprising DNA operably linked to a promoter compatible with a suitable host cell, said DNA encoding a chimeric immunoglobulin heavy or light chain having specificity for a particular known antigen and having a constant region homologous to a corresponding region of an antibody of a first mammalian species and a variable region homologous to a variable region of an antibody derived from a second, different mammalian species.

6. The vector of claim 5 wherein the first mammalian species is human.

7. Recombinant host cells transformed with the vector of claim 5.

* * * * *

EXHIBIT G

A Tissue-specific Transcription Enhancer Element Is Located in the Major Intron of a Rearranged Immunoglobulin Heavy Chain Gene

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Summary

We have studied the DNA sequences required for high level expression of a cloned heavy chain immunoglobulin gene stably introduced into mouse myeloma cells by DNA transfection. We found that DNA sequences derived from the germ line J_H–C_μ region are required for accurate and efficient transcription from a functionally rearranged V_H promoter. Similar to viral transcriptional enhancer elements, these cellular sequences stimulate transcription from either the homologous V_H gene segment promoter or a heterologous SV40 promoter. They are active when placed on the 5′ or 3′ side of the rearranged V_H gene segment and they function when their orientation is reversed. However, unlike viral enhancers, the Ig gene enhancer appears to act in a tissue-specific manner, since it is active in mouse B cells but not in mouse fibroblasts. The nucleotide sequence of the Ig enhancer region contains repeating elements that closely resemble sequence elements found in many viral enhancers. We discuss the possible role of tissue-specific transcription in cell differentiation and malignant transformation.

Introduction

Molecular analyses of immunoglobulin (Ig) genes established that an Ig polypeptide chain is encoded in multiple gene segments scattered along a chromosome of the germ line genome and that these gene segments must be brought together to form a complete Ig gene active in B lymphocytes (For a review see Tonegawa, 1983) This somatic assembly of Ig gene segments is achieved by a series of developmentally-controlled recombination events that occur during the differentiation of B cells

Two types of recombination occur V–J or V–D–J joining and switch recombination. The V–J and V–D–J joinings are essential for the formation of the DNA sequence coding for the variable (V) region of the light and heavy chain, respectively, while switch recombination replaces the constant (C) region-coding sequence of the heavy chain of one class (usually μ) with another (γ, ϵ, α, etc) The exact timing of these recombination events in the course of B cell development has not been determined, but both V–J and V–D–J joining occur before the cell encounters antigens

One of the most important functions of the V–J and V–D–J joinings is to create complete Ig genes with a diverse set of V region-coding DNA sequences from a limited number of the gene segments carried in the germ line genome (Tonegawa, 1983) Another important function of V–J and V–D–J joining events is in the control of the expression of Ig genes during B cell differentiation On the basis of the fine structural analysis of Ig genes, it is clear that these joining events are prerequisite for the synthesis of a complete Ig chain there is no evidence that any unrearranged germ line Ig gene segment can directly participate with its coding capacity in the synthesis of a complete, functional Ig chain (Brack et al, 1978, Bernard et al., 1978, Sakano et al., 1979, Max et al, 1979, Early et al., 1980, Sakano et al., 1980) In fact, it has been shown that in a myeloma cell the RNA transcript of an unrearranged V, segment is no more than 0 1 copy per gene, a level at least four orders of magnitude lower than that of the transcript from the rearranged, expressed V, segment present in the same cell (Mather and Perry, 1981)

Both the V–J and V–D–J joining events alter the sequence configurations in the 3′ region of the germ line V gene segment, but the 5′ flanking region of the V gene segment, where the transcription promoter and other controlling elements reside, is unaffected by the rearrangement (Bernard et al, 1978 and Clarke et al, 1982) Although a few nucleotide differences have been found between the germ line and somatic sequences in the 5′ flanking region of V gene segments (Sakano et al, 1980), these base substitutions are by-products of the somatic mutation events whose physiological role is to diversify the V-coding sequences (Bernard et al, 1978, Weigert and Riblet, 1976, Selsing and Storb, 1981) These base changes are not systematic and therefore are thought to have no bearing on the control of Ig gene expression (Clarke et al., 1982)

A possible explanation of how the downstream sequence might confer transcriptional competence to the rearranged V gene segment promoter is through transcriptional enhancement Although the mechanism of this phenomenon is unknown, specific viral DNA sequence elements have been described (Banerji et al, 1981, de Villiers et al, 1981, Levinson et al, 1982) which enhance viral or recombinant cellular gene transcription Because such enhancer elements can activate transcription from promoters which are located either upstream or downstream, and more than 1 kb away, it is possible that an analogous element might be located near the C gene segment The observations that the C gene segments, in contrast to the V gene segments, are transcriptionally active in lymphoid cells in the absence of rearrangement support this hypothesis In this case, promoter-like sequences upstream of the C gene segments are utilized for transcription but the

transcripts are degraded in the nuclei (Kemp et al , 1980, Van Ness et al , 1982)

The recent technical advances for introducing cloned immunoglobulin genes into lymphoid cells (Oi et al , 1983, Rice et al., 1983) have made it possible to study the structure-function relationship between specific DNA sequences and gene expression in these cells We describe an enhancer element in the major intron of a rearranged γ_{2b} heavy chain gene This sequence is located between the J_H region and the switch-recombination site utilized in myeloma MOPC 141, i.e., it is derived from sequences upstream of the germ line C_μ gene segment

Results

High Level Expression of the Heavy Chain Gene Introduced into Myeloma Cells

We previously reported that the functionally rearranged immunoglobulin heavy chain (γ_{2b}) gene from MOPC 141 tumor cells (Sakano et al , 1980) can be accurately expressed at a low level in transfected mouse L cells (Gillies et al , 1983) For the studies presented here, we subcloned the same γ_{2b} gene fragment into plasmid pSV2gpt (Mulligan and Berg, 1980), transfected the mouse myeloma line, J558L, and selected for gpt gene activity by resistance to mycophenolic acid. This J558L line has lost the ability to express the endogenous immunoglobulin heavy chain gene but continues to synthesize a λ light chain Furthermore, J558L has been shown to have a relatively high transformation frequency ($>10^{-4}$) when pSV2gpt vectors containing light chain genes are used for transfection (Oi et al., 1983)

Using a modified protocol for protoplast fusion (see Experimental Procedures), we found that plasmid pSV-γ_{2b}VC (Figure 1) transforms J558L cells at a frequency of greater than 10^{-3}. This high frequency made it possible to use pools of independently-derived clones of gpt transformants to compare the expression of plasmids containing defined deletions with that of the parental plasmid pSV-γ_{2b}VC The advantage of this method is that the resulting cell lines represent several independent integration events (required for transformation), therefore the level of heavy chain gene expression in a given pool should reflect the average level of the individual clones Thus the possible effect of the site of integration on the expression of the transfected gene is minimized

Cell lines obtained by transfection with plasmid pSV-γ_{2b}VC and selection for gpt expression (growth in the presence of mycophenolic acid) were found to express high levels of γ_{2b} heavy chain (Figure 2A, lanes 2-5) These levels of expression of the exogenous γ_{2b} genes are estimated to be about 20% of that of the endogenous γ_{2b} gene in MOPC 141 Apparently, this heavy chain can form an immunoglobulin molecule with the λ light chain of myeloma J558L, because the light chain was immunoprecipitated from cell extracts with antiheavy chain antisera and equimolar amounts of heavy and light chain were secreted into the culture medium (Figure 2A, lane 14)

A Deletion of Part of the Major Intron Abolishes the High Level Expression of the Heavy Chain Gene

Deletion mutants of the parental plasmid were constructed to test whether the removal of specific noncoding DNA sequences would affect the expression of the γ_{2b} gene in J558L cells Because deletions between the VDJ and C_μ exons of an Abelson murine leukemia virus transformed cell line have been correlated with decreased heavy chain production (Alt et al , 1982), we constructed mutant plasmids with deletions in this region Two such plasmids, pSV-γ_{2b}3'RΔ1 and pSV-γ_{2b}3'RΔ2 contain overlapping deletions around the unique Eco RI site of the parental plasmid pSV-γ_{2b}VC (Figure 1) These three plasmids were introduced into J558L myeloma cells and the expression of the γ_{2b} heavy chain gene in stably transformed cells was compared

Cell lines obtained by transfection with plasmid pSV-γ_{2b}3'RΔ1 synthesized high levels (no less than half of the wild type level) of γ_{2b} heavy chain (Figure 2A, lanes 6-9) and secreted immunoglobulin (Figure 2A, lane 15) In contrast, four cell lines obtained by transfection with plasmid pSV-γ_{2b}3'RΔ2 synthesized only low levels (about 5% of the wild type level) of heavy chain (Figure 2A, lanes 10-13) The same results were obtained when subclones of
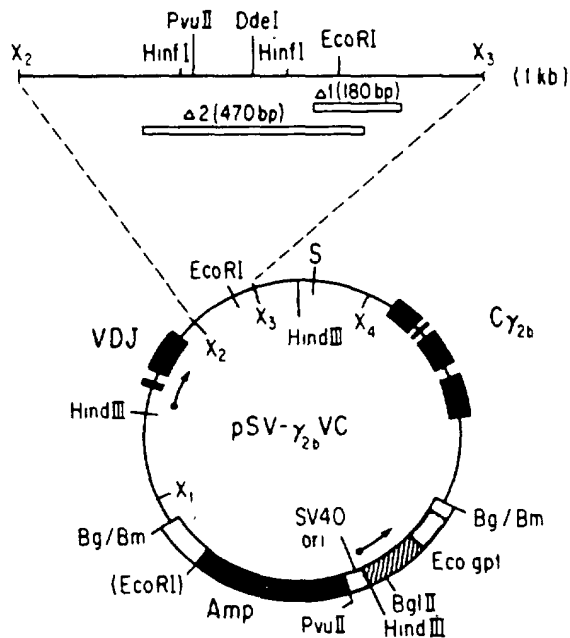
Figure 1 Partial Restriction Map of Plasmid pSV γ_{2b}VC

A 9 kb Bgl II fragment was inserted into the Bam HI site (indicated by Bg/Bm) of plasmid pSV2gpt The Ecogpt gene (wide stripes) is flanked by SV40 sequences (thin stripes) including the origin (ori) of replication and mRNA start site (arrow) The γ_{2b} gene (narrow line) contains VDJ and $C_{\gamma 2b}$ exons (solid boxes) and a mRNA start site (arrow) about 30 bp upstream of the VDJ coding sequence (Gillies and Tonegawa 1983) The switch recombination (S) site is also shown The DNA segments deleted in plasmids pSV γ_{2b}3'RΔ1 and pSV γ_{2b}3'RΔ2 are shown in linear form above the circular map The sizes of the deletions, as determined by restriction analysis are indicated The exact locations of these deletions are shown in Figure 7

A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M

γ_{2b} -

λ -

— 69K
— 46K
— 30K
— 14K

C $\overbrace{\gamma_{2b}VC}$ $\overbrace{\Delta 1}$ $\overbrace{\Delta 2}$ $\overbrace{Secreted}$

B

1 2 3 4 5 6 7 8 9 10 11 M

γ_{2b} -

λ -

— 69K
— 46K
— 30K
— 14K

$\overbrace{\gamma_{2b}VC}$ $\overbrace{\Delta 1}$ $\overbrace{\Delta 2}$ C
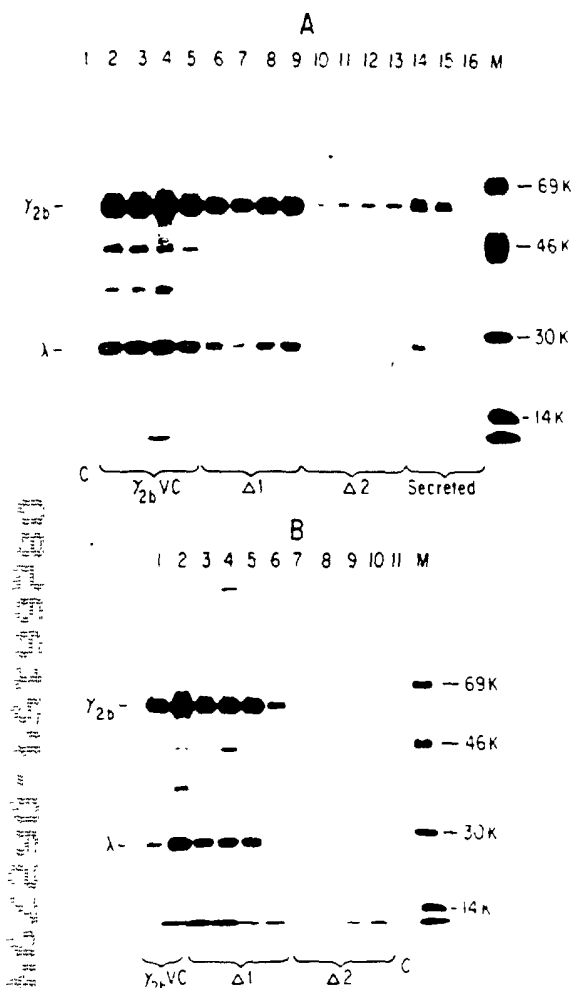
Figure 2 Expression of γ_{2b} Heavy Chain Protein in Transfected Cells

Transfected cell lines were labeled with ^{35}S-methionine and cell extracts were analyzed as described in Experimental Procedures Immunoprecipitated proteins were analyzed by SDS polyacrylamide gel electrophoresis and fluorography (A) Four transfected lines (pools of individual clones) were analyzed for each plasmid tested Lane 1 control J558L cells, lanes 2–5 cells transfected with plasmid pSV γ_{2b}VC, lanes 6–9 cells transfected with plasmid pSV γ_{2b}3'RΔ1 lanes 10–13 cells transfected with plasmid pSV γ_{2b}3'RΔ2 Secreted proteins from cells transfected with plasmid pSV γ_{2b}VC (lane 14), plasmid pSV γ_{2b}3'RΔ1 (lane 15), and plasmid pSV γ_{2b}3'RΔ2 (lane 16) were immunoprecipitated and analyzed on the same gel (B) Cell lines subcloned from the transfected cell lines were tested for γ_{2b} heavy chain protein synthesis as in (A) The plasmids used for transfection are indicated below the autoradiogram Control (C) cell extract is shown in lane 11 The positions of the γ_{2b} heavy chain and λ light chain (synthesized in J558L cells but not immunoprecipitated in the absence of γ_{2b} heavy chain) are indicated

each pool were tested for γ_{2b} heavy chain expression (Figure 2B), although more variation was observed in the level of expression between individual clones Nonetheless, these results strongly suggest that DNA sequences deleted in plasmid pSV-γ_{2b}3'RΔ2, but still present in pSV-γ_{2b}3'RΔ1, are essential for the high level expression of heavy chain genes in myeloma cells

1 2 3 4 5 6 7 8

3.4 -

1.7 -
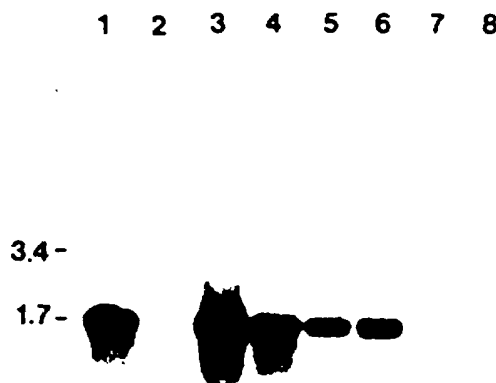
Figure 3 Northern Gel Blotting Analysis of Transfected Cell RNA

Total cell RNA (10 μg per lane) was electrophoresed on a denaturing agarose gel transferred to nitrocellulose and hybridized to nick translated Cγ_{2b} probe RNA (2 5 μg) from MOPC 141 cells (lane 1) and J558L cells (lane 2) were also analyzed Two cell lines transfected with plasmid pSV γ_{2b}VC (lanes 3 and 4) plasmid pSV γ_{2b}3'RΔ1 (lanes 5 and 6) and plasmid pSV γ_{2b}3'RΔ2 (lanes 7 and 8) are shown The positions of the secreted (1 7 kb) and membrane forms (3 4 kb) of γ_{2b} mRNA are indicated

The Reduced Expression of the γ_{2b} Gene Is at the Level of RNA

The steady-state level of γ_{2b} mRNA in transfected cell lines was analyzed by Northern gel blotting and hybridization with the Cγ_{2b} probe It should be noted that the γ_{2b} heavy chain gene used in these studies does not contain the exons coding for the membrane form of γ_{2b} (Gillies and Tonegawa, 1983) and thus the only species of mRNA expected in transfected cells is the secreted form (1 7 kb)

As seen in Figure 3, cell lines transfected with plasmid pSV-γ_{2b}VC (lanes 3 and 4) and plasmid pSV-γ_{2b}3'RΔ1 (lanes 5 and 6) contain high levels of the secreted form of γ_{2b} mRNA The cell lines transfected with plasmid pSV-γ_{2b}3'RΔ2 (Figure 3, lanes 7 and 8) contained much lower levels of γ_{2b} mRNA of the correct size, in agreement with the decreased level of γ_{2b} heavy chain protein (Figure 2) The additional RNA bands seen in lanes 7 and 8 (Figure 3) also contain γ_{2b} sequences but appear to be read-through products of the Ecogpt gene Data presented below support this explanation, as opposed to the idea that the intron deletion has a deleterious effect on RNA splicing and results in low levels of translatable γ_{2b} mRNA

Plasmid Copy Number in Transfected Cell Lines

DNA from transfected cells was analyzed by Southern gel blotting to determine the plasmid copy number and its possible effect on the level of γ_{2b} mRNA When a pSV2gpt plasmid DNA probe was used for hybridization, a striking difference in plasmid copy number was found (Figure 4) Two prominent bands, corresponding to the two large Hind III fragments (6.2 kb and 5.0 kb) common to all the plasmids, are detected with this probe (seen best in Figure 4, lanes 6 and 7) Clearly, the DNA sequences deleted in plasmid pSV2-γ_{2b}3'RΔ2 (those required for the high level expression of γ_{2b} mRNA) have a dramatic effect on the number of copies of plasmid required for transformation to the gpt⁺ phenotype When these sequences are present, as they are in plasmids pSV-γ_{2b}VC and pSV-γ_{2b}3'RΔ1, a low copy number is sufficient for gpt transformation (Figure 4, lanes 2-5). In the absence of these sequences, the copy number is increased at least 20-fold (Figure 4, lanes 6 and 7), presumably to compensate for a comparable decrease in gpt mRNA transcription.

Two conclusions can be made from these results: one, the DNA sequences required for the high level expression of γ_{2b} mRNA also increase the level of expression from the heterologous SV40 promoter at least 20 times, two, the level of RNA transcribed from the V gene segment promoter is decreased about 400 times per gene copy in the absence of this DNA sequence This calculation is based on the observed decrease by a factor of 20 in γ_{2b} gene expression as a result of the 3'RΔ2 deletion, and the fact that this decreased level is likely the result of the transcription of at least 20 times as many gene copies

DNA Sequences Located in the γ_{2b} Gene Intron Enhance Expression in an Orientation-independent and a Position-independent Manner

The DNA sequences defined as viral enhancer elements have been shown to stimulate the transcription of homologous or heterologous promoters either upstream or downstream, and in either orientation with respect to the direction of transcription (Moreau et al 1981, Wasylyk et al, 1983). In order to test whether the sequences located in the major intron of the γ_{2b} gene (and all other heavy chain genes) behave similarly, we constructed a plasmid with most of the intron sequences deleted. We then inserted a 1 kb Xba I fragment ($X_{2/3}$) containing those intron sequences with potential enhancer activity into either of two sites in either of the two orientations The first corresponds to the original position of this fragment in the parental plasmid (as part of the VDJ-Cγ_{2b} intron) and the second is approximately 1 4 kb upstream (on the 5' side of the V gene segment) Four plasmids were obtained which contained the $X_{2/3}$ fragment in the normal or reversed orientation, either upstream or downstream of the mRNA start site (see Figure 5A)

Cell lines obtained by transfection with the plasmids just described were analyzed for the expression of γ_{2b} heavy chain As seen in Figure 5B, cells transfected with plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$ (with most of the intron deleted) did not
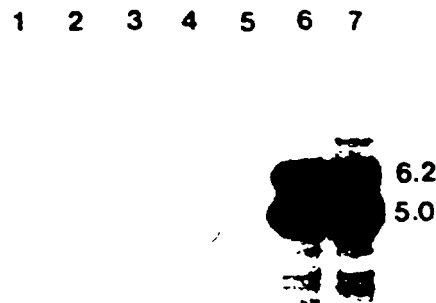
Figure 4 Southern Gel Blotting Analysis of Plasmid DNA Sequences in Transfected Cell Lines

High molecular weight DNA (10 μg per lane) from J558L cells (lane 1), or cell lines transfected with plasmid pSV γ_{2b}VC (lanes 2 and 3), plasmid pSV γ_{2b}3'RΔ1 (lanes 4 and 5) or plasmid pSV γ_{2b}3'RΔ2 (lanes 6 and 7) was digested with Hind III, electrophoresed on a 0 8% agarose gel, transferred to nitrocellulose and hybridized to nick translated pSV2gpt DNA The positions of the two Hind III fragments (6 2 kb and 5 0 kb) common to all the transfected plasmid DNAs (both of which hybridize to the pSV2gpt probe), are indicated

synthesize significant levels of γ_{2b} protein (lanes 3 and 4) The insertion of the $X_{2/3}$ fragment into the intron site (the normal position of this fragment) restored the expression of γ_{2b} protein in both the normal (Figure 5B, lanes 5 and 6) or reversed (Figure 5B, lanes 7 and 8) orientations Similarly, insertion of the same fragment upstream of the V gene segment (on the 5' side of the transcriptional promoter) in either the normal (Figure 5B, lanes 9 and 10) or the reversed (Figure 5B, lanes 11 and 12) orientation also restored the expression of γ_{2b} protein to normal levels.

These results clearly demonstrate that the intron sequences deleted in the 3'RΔ2 mutant plasmid have a direct effect on transcription in a manner that is analogous to the viral enhancers They also show that the enhancer function does not require the expression of these sequences in the γ_{2b} gene primary transcript, because movement of the $X_{2/3}$ fragment outside of the transcription unit (i.e , the Xba I_1 site) had no effect on its ability to function.

Tissue Specificity of the Immunoglobulin Enhancer Element

The rearranged γ_{2b} gene used in these studies is also accurately transcribed in mouse fibroblasts (Ltk⁻ cells)

A.

B.
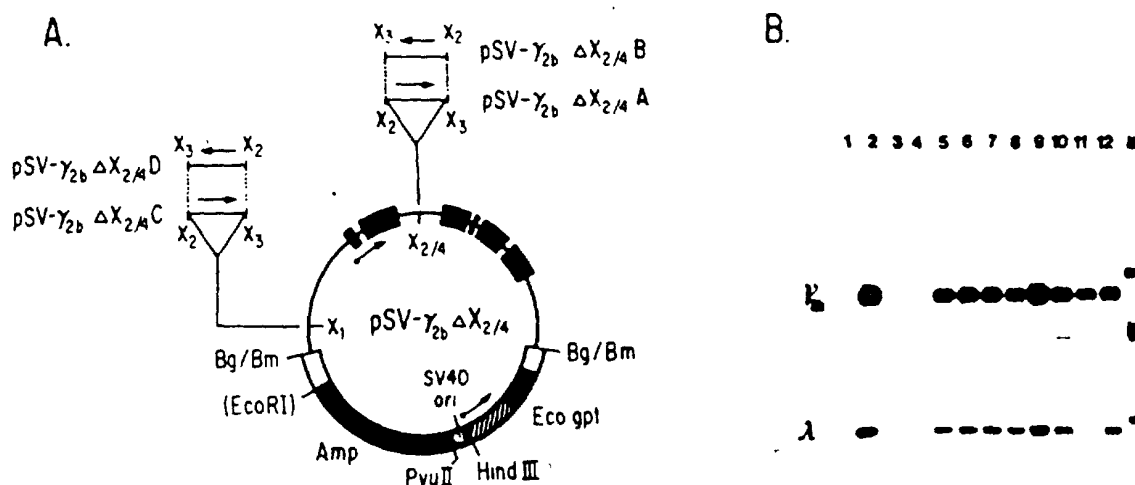
Figure 5. Enhancement of γ_{2b} Gene Expression by a 1 kb Intron Fragment

(A) Restriction map of plasmid pSV $\gamma_{2b}\Delta X_{2/4}$. This plasmid was constructed from plasmid pSV γ_{2b}VC (shown in Figure 1) by removing two Xba I fragments from the γ_{2b} gene intron (from the X_2 to the X_4 sites in Figure 1). Derivatives of plasmid pSV·$\gamma_{2b}\Delta X_{2/4}$, labeled A through D, contain inserts of the 1 kb $X_{2/3}$ fragment in the sites indicated and the orientation (relative to transcription) is shown with an arrow. (B) Expression of γ_{2b} heavy chain in cells transfected with the plasmids shown in (A). Analysis was carried out as described in Figure 2. Cell lines tested were J558L (lane 1) and those transfected with plasmid pSV·γ_{2b}VC (lane 2), plasmid·pSV2·$\gamma_{2b}\Delta X_{2/4}$ (lanes 3 and 4), plasmid pSV·$\gamma_{2b}\Delta X_{2/4}$A (lanes 5 and 6), plasmid pSV·$\gamma_{2b}\Delta X_{2/4}$B (lanes 7 and 8), plasmid pSV·$\gamma_{2b}\Delta X_{2/4}$C (lanes 9 and 10), and plasmid pSV·$\gamma_{2b}\Delta X_{2/4}$D (lanes 11 and 12)

cotransfected with the herpes virus tk gene and plasmid ppLγ_{2b}VC (Gillies and Tonegawa, 1983) The level of γ_{2b} gene expression in these cells was found to be proportional to the number of transfected genes, but is at least two orders of magnitude less per gene copy than in myeloma cells Thus it is likely that the enhancer element, described above, does not function in nonlymphoid cells

In order to compare the levels of γ_{2b} gene expression in fibroblasts, with and without the immunoglobulin enhancer, we first made a deletion mutant, ppL$\gamma_{2b}\Delta X_{2/3}$, lacking these sequences (Figure 6A) We then modified the plasmids so that a high copy number of γ_{2b} genes would be integrated into the transfected L cells and increase the expression of the γ_{2b} gene to an easily detectable level This was done by inserting a truncated tk gene (a 2.3 kb Eco RI fragment containing only limited 5′ upstream sequences) into both the wild type and mutant plasmids. Transformation to the tk⁺ phenotype with this fragment requires the transfer of multiple plasmid copies into cells, thus another gene on the same plasmid would also be present at a high copy number in tk⁺ transformants (our unpublished results).

Plasmids ppLγ_{2b}·TK and ppL$\gamma_{2b}\Delta X_{2/3}$·TK were introduced into mouse Ltk⁻ cells and the tk⁺ transformants (approximately 50 individual clones) were pooled, grown in mass culture, and tested for the presence of γ_{2b} DNA sequences As seen in Figure 6B, each transfected cell line contained comparable numbers of tandem, head-to-tail oligomers of either plasmid. Control experiments (not shown) indicate that individually cloned cell lines also contain the same number (about 15 copies per cell) of transfected plasmid DNA Apparently the copy number is determined by the level of expression of the tk gene which,

in this case, has been reduced considerably by the deletion of the upstream sequences To compensate for the low level of expression, multiple copies of the tk gene are required for tk transformation This, then, is analogous to the results with pSV2gpt vectors described above

We compared the expression of the normal and mutant γ_{2b} heavy chain genes in these cell lines by Northern gel blotting analysis of total cell RNA As seen in Figure 6C, the steady-state level of γ_{2b} mRNA is not affected by the deletion of the immunoglobulin enhancer We concluded that the low level expression of the heavy chain gene in L cells is a result of the fact that this enhancer element is functional only in lymphoid cells

Additional experiments have been carried out to test the tissue specificity of the immunoglobulin enhancer We constructed a derivative (pSER) of plasmid pSV2gpt lacking most of the SV40 72 bp repeat sequence (see Experimental Procedures) When this plasmid is used to transfect either mouse L cells or J558L myeloma cells, the transformation frequency (relative to that of plasmid pSV2gpt) is lowered by more than a factor of 20 (from 2×10^{-3} to 10^{-4} in L cells and from 3×10^{-4} to 10^{-5} in J558L myeloma cells—Table 1) When the 1 kb $X_{2/3}$ fragment containing the immunoglobulin enhancer is inserted into the Eco RI site of plasmid pSER, the transformation frequency is restored to the level of plasmid pSV2gpt, but only in myeloma cells There is no effect on the transformation frequency of plasmid pSER in L cells (Table 1) Thus the enhancing effect on the heterologous SV40 promoter (which controls the Ecogpt gene) is also tissue-specific

Using this same transformation assay we tested smaller restriction fragments for enhancer activity A 140 bp Pvu II-Dde I fragment (see Figure 1), containing some of the
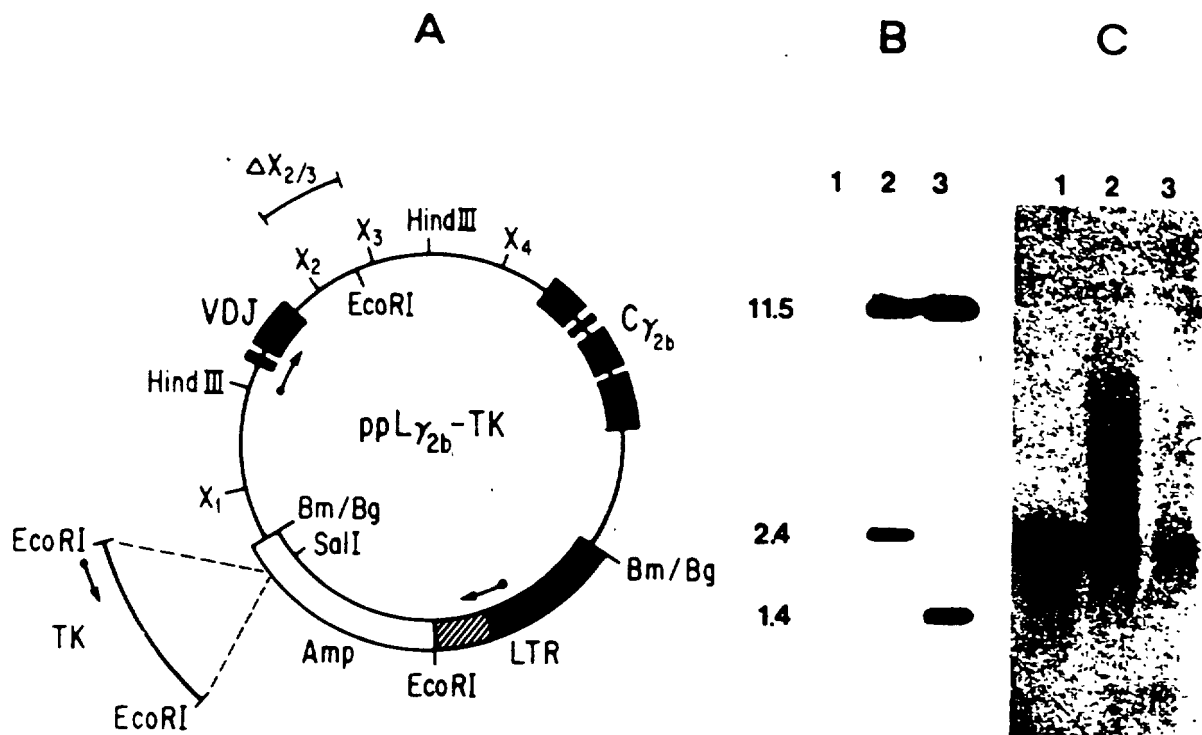
Figure 6   Expression of γ$_{2b}$ mRNA in Mouse L Cells Transfected with Plasmids ppLγ$_{2b}$ tk and ppLγ$_{2b}$·ΔX$_{2/3}$·tk

(A) Restriction map of the plasmids used for transfection Plasmid ppLγ$_{2b}$ tk was constructed by inserting a 2 3 kb fragment of the herpes virus tk gene into the unique Sal I site of plasmid ppLγ$_{2b}$VC (Gillies and Tonegawa 1983) The direction of transcription of the tk gene (arrow) is opposite that of the γ$_{2b}$ gene The sequences deleted in plasmid ppLγ$_{2b}$ΔX$_{2/3}$ tk are indicated (B) Southern gel blotting analysis of DNA from L tk⁻ cells (lane 1), and cells transfected with plasmid ppLγ$_{2b}$ tk (lane 2) or plasmid ppLγ$_{2b}$ΔX$_{2/3}$ tk (lane 3) DNA was digested with Hind III and hybridized to nick translated γ$_{2b}$ DNA (the 9 kb Bgl I fragment used for plasmid construction) (C) Northern gel blotting analysis of total cell RNA from MOPC 141 myeloma cells (lane 1), and cells transfected with plasmid ppLγ$_{2b}$ tk (lane 2), or plasmid ppLγ$_{2b}$ΔX$_{2/3}$ tk (lane 3) Nick translated Cγ$_{2b}$ probe (Gillies and Tonegawa 1983) was used for hybridization

sequences deleted in plasmid pSV-γ$_{2b}$3'RΔ2, was found to increase the transformation frequency of plasmid pSER by 20-fold in J558L cells but not in mouse L cells (Table 1) Thus we have localized the immunoglobulin enhancer sequence to this portion of the X$_{2/3}$ fragment

DNA Sequences in the Heavy Chain Gene Intron Resemble Viral Enhancers

Weiher et al (1983) have suggested that the sequence 5′ GTGG$^{AAA}_{TTT}$G3′ (where ^A_T means either A or T appears at that position) represents a crucial core element common to all of the known viral enhancers Sequence analysis of the X$_{2/3}$ fragment (the 1 kb fragment shown to have enhancer activity—Figure 5) shows that such a sequence is located in the region that is deleted in plasmid pSV·γ$_{2b}$3'RΔ2 (but not in the 3'RΔ1 mutant) and is present in the 140 bp Pvu II–Dde I fragment In fact, the sequence 5'GTGGTTT(T)GAA·3' is present as a closely spaced repeat (Figure 7), oriented in the direction of transcription The first eight nucleotides of this sequence are also found upstream of the tandem repeat, but oriented in the opposite direction

Figure 8 shows a comparison of several viral sequences shown to have enhancer activity and the repeat sequences

Table 1   Transformation Frequency of pSV2gpt and Derivative Plasmids in J558L Myeloma Cells and L Cells

Plasmid	Cell Type	
	J558L	L Cell
pSV2gpt	3×10^{-4}	2×10^{-3}
pSER	8×10^{-6}	1×10^{-4}
pSER X$_{2/3}$	4×10^{-4}	1×10^{-4}
pSER X$_{2/3}$(140)	2×10^{-4}	9×10^{-5}

Cells were transfected by protoplast fusion and plated at 10^4 cells per well and 2×10^3 cells per well (J558L) or at 10^4 and 10^5 cells per 100 mm dish (L cells) Selective medium containing mycophenolic acid (6 μg/ml for J558L or 25 μg/ml for L cells) was added at 48 hr and colonies were counted at 10 days (J558L) or at 14 days (L cells) Derivatives of plasmid pSER were constructed by inserting (blunt end ligating) either the 1 kb X$_{2/3}$ fragment or a 140 bp Pvu II-Dde I [X$_{2/3}$(140)] fragment (see Figure 1) into the Eco RI site

in the γ$_{2b}$ gene intron Sequences contained in the Moloney sarcoma virus (MSV) 73 bp repeat sequence appear to be most similar to the immunoglobulin sequence, especially on the 5′ side of the first "core" repeat The "core" sequence of polyoma virus was most similar to the second "core" repeat, as both contain an additional T residue

Also shown are two sequences, present in the immu

Xba I   50   100

TCTAGAGAGG TCTGGTGGAG CCTGCAAAAG TCCAGCTTTC AAAGGAACAC AGAAGTATGT GTATGGAATA TTAGAAGATG TTGCTTTTAC TCTTAAGTTG

150   200

GTTCCTAGGA AAAATAGTTA AATACTGTGA CTTTAAAATG TGAGAGGGTT TTCAAGTACT CATTTTTTTA AATGTCCAAA ATTTTTGTCA ATCAATTTGA

Δ2 ⌐250   300

GGTCTTGTTT GTGTAGAACT GACATTACTT AAAGTTTAAC CGAGGAATGG GAGTGAGGCT CTCTCATACC CTATCCAGAA CTGACTTTTA ACAATAATAA

Hinf I 350   Pvu II   400

ATTAAGTTTA AAATATTTTT AAATGAATTG AGCAATGTTG AGTTGAGTCA AGATGGCCGA TCAGAACCAG AACACCTGCA GCAGCTGGCA GGAAGCAGGT

◀   450   ◆   ◆   500

CATGTGGCAA GGCTATTTGG GGAAGGGAAA ATAAAACCAC TAGGTAAACT TGTAGCTGTG GTTTGAAGAA GTGGTTTTGA AACACTCTGT CCAGCCCCAC

Dde I   550   Hinf I   600

CAAACCGAAA GTCCAGGCTG AGCAAAACAC CACCTGGGTA ATTTGCATTT CTAAAATAAG TTGAGGATTC AGCCGAAACT GGAGAGGTCC TCTTTTAACT

Δ1 ⌐650   EcoRI   700

TATTGAGTTC AACCTTTTAA TTTTAGCTTG AGTAGTTCTA GTTTCCCCAA ACTTAAGTTT ATCGACTTCT AAAATGTATT TAGAATTCAT TTTCAAAATT

⌐Δ2   750   800

AGGTTATGTA AGAAATTGAA GGACTTTAGT GTCTTTAATT TCTAATATAT TTAGAAAACT TCTTAAAATT ACTCTATTAT TCTTCCCTCT GATTATTGGT

⌐Δ1   850   900

TCCATTCAA TTATTTTCCA ATACCCGAAG TCTTTACAGT GACTTTGTTC ATGATCTTTT TTAGTTGTTT GTTTTGCCTT ACTATTAAGA CTTTGACATT

Dde I 950   Xba I

CTGGTCAAAA CGGCTTCACA AATCTTTTTC AAGACCACTT TCTGAGTATT CATTTTAGGA GAAATATTTT TTTTTTAAAT GAATGCAATT ATCTAGA

Figure 7 Nucleotide Sequence of the 1 kb X₂ₐ Fragment

DNA sequencing was carried out according to standard procedures (Maxam and Gilbert, 1980) The sequences deleted in plasmids pSV γ₂ₐ3'RΔ1 and pSV γₐ3'RΔ2 are indicated The underlined sequences are those similar to the "core" elements common to most viral enhancers (Weiher et al 1983) Arrows indicate the orientation of the immunoglobulin "core" elements relative to the direction of γ₂ₐ mRNA transcription

```
                    TTT
              GTGG     G
                  AAA
                                                    "Core"
      ◀              ◆           ◆
   AATAAAACCACTAGGTAAACTTGTAGCTGTGGTTTGAAGAAGTGGTTTTGAAACACTCTGT
  430                                               490        Ig Cμ

         ****  *** **********  *      *
      AAACAGGTATCTGTGGTAAGCGGTTCCTGCCCCG                        MSV
     130        A              165

      GAGGGCGTGTGGTTTGAAGAGG                                    Polyoma
     5180        TC      5203

             GAGGGCGTGTGGTTTTGCAAGAGG                           Polyoma
           5180              5203

         GTTAGGGTGTGGAAAGTCCCCAGG                               SV40
        238              215

       TTGTTTTTCTTGGTAAGAACTCTCAGTTTCTGTTTT                     Ig Cκ
      3863         A              3899

       AGATGGCTCTCGGAAAGTCCCCTCTGTTGAGATGCC                     Ig Cκ
      3958                     3923

          AATCAAGAGCTGGAAAGAGAGGGTCAGGTGTCTGTA                  Ig Cλ₁
         925                     961
```

Figure 8 A Comparison of Sequences in the 1 kb X₂ₐ Fragment with Those of Viral Enhancer Elements

Sequences are aligned at the putative "core" (underlined) sequences (Weiher et al, 1983) The residues which match those in the immunoglobulin heavy chain sequence are indicated by an asterisk These include either an A or T residue in positions 5-7 of the "core" sequence In some cases a residue has been displaced to maximize the homology Numberings of the sequences are according to Van Beveren et al 1981 (MSV) Griffin et al 1980 (Polyoma) Buchman et al 1980 (SV40) Max et al 1981 (IgC,) Bernard and Tonegawa unpublished data (Ig Cλ₁)

noglobulin light chain gene intron, that resemble an enhancer element The existence of such a sequence near the C, gene segment is rendered plausible by its proximity to a DNAase I hypersensitive site (Parslow and Granner, 1982) In addition, studies using transfected genes suggest that deletions in this region reduce transcription from the V promoter (V O and S M, unpublished data) A similar core-like sequence is also present near the constant portion of the λ, light chain gene, but there is yet no evidence showing that this is part of an enhancer element

Discussion

Evidence for an Enhancer Element in the Intron of a Heavy Chain Immunoglobulin Gene

Sequences contained in the major intron between the functionally rearranged VDJ and C exons of a heavy chain immunoglobulin gene were shown to be essential for its high level expression in transfected myeloma cells Although the deletion of these sequences decreases the level of expression in transfected cells 20 times (Figure 2), the actual reduction is probably about 400 times per gene copy We have tested whether these sequences are analogous to viral enhancer elements In addition to increasing the level of transcription from homologous promoters, viral enhancers also increase transcription from many heterologous, viral or nonviral promoters This enhancing activity is independent of the orientation of the enhancer element, relative to the direction of transcription, and is independent of its position as long as the distance between the enhancer and promoter is within several kilobases (Banerji et al , 1981, Moreau et al , 1981, Wasylyk et al., 1983)

These properties also apply to the sequences contained in the heavy chain immunoglobulin gene Enhancement of the heterologous SV40 promoter occurs when the intact γ_{2b} heavy chain gene is present in plasmid pSV2gpt and a low copy number of the recombinant plasmid is sufficient for gpt transformation When the γ_{2b} gene intron sequences are removed, the plasmid copy number increases dramatically to compensate for the decreased expression of the gpt gene from the SV40 promoter (Figure 4)

We also found that DNA fragments from the γ_{2b} gene intron can substitute for the SV40 enhancer in plasmid pSV2gpt (Table 1) This transformation assay is based on the ability of the DNA fragments, located more than 2 kb away from the SV40 promoter, to enhance the transcription of the gpt gene and thereby increase the transformation

frequency Using this method we have shown that most of the enhancing activity can be localized to a 140 bp fragment The nucleotide sequence in this region contains a repeat sequence which closely resembles the "core" nucleotides found in most viral enhancers (Figure 8)

Finally, we demonstrated that this intron sequence maintains its ability to stimulate transcription of the heavy chain gene when it is moved outside of the γ_{2b} transcription unit (5' of the mRNA start site) and when its orientation is reversed (Figure 5) These results show that the immunoglobulin intron sequence is an enhancer element and has properties in common with those of viral origin, even though the latter are generally located on the 5' side of their cognate transcriptional promoters

The Role of Transcriptional Enhancement in the Regulation of Immunoglobulin Gene Expression

The creation of active immunoglobulin genes through somatic recombination has been studied in detail (reviewed by Tonegawa, 1983), but the mechanism by which this activation is brought about has been a major problem of molecular immunology The observation that the C_μ and C_κ gene segments are transcriptionally active in lymphoid cells, prior to V-J or V-D-J joining (Kemp et al., 1980, Van Ness et al , 1982), provided the first evidence that sequences downstream of the V gene segment promoter might affect the transcription of the functionally rearranged gene

The mechanism of this activation can now be explained, at least for the heavy chain gene, by our identification of an enhancer element between the J_H and $C\gamma_{2b}$ gene segments of a functionally rearranged gene This site corresponds to the J_H-C_μ region of germ-line DNA (see Figure 9) Following VDJ-joining, which occurs before B cells encounter antigens, this enhancer (which would now be part of the major intron of the functionally rearranged μ
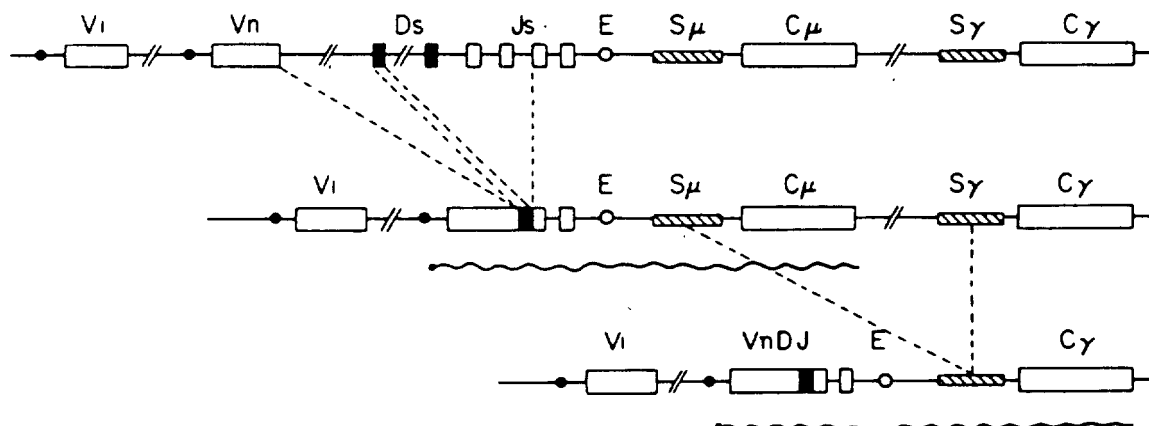
Figure 9 Schematic Diagram Showing Arrangements of Various Ig Heavy Chain Gene Segments and the Position of the Enhancer

Top, middle and bottom arrangements correspond to germ-line DNA, μ chain positive preswitch B cell DNA, and γ chain positive, postswitch plasma cell DNA, respectively The enhancer element (O) is located between the J_H segments and C_μ segments in the germ-line DNA and becomes part of the major intron in the active μ chain gene upon V-D-J joining The same enhancer element is retained in the major intron of the active γ chain gene which is created by a switch recombination from the μ gene S_μ and S_γ refer to the regions in which switch recombinations occur The wavy lines and the large filled circles (●) represent the primary transcripts and the promoters respectively The small filled circles (•) represent the 5' caps of the RNA molecules

chain gene) activates the promoter of the rearranged V gene segment. In this way only a single V gene segment (out of several hundred) would be transcriptionally active, and only after functional rearrangement had occurred

Subsequent to the encounter with antigens and stimulation by T cells, a second type of rearrangement (switch recombination) occurs in heavy chain genes and results in the replacement of the C_μ coding sequence (Figure 9) with those of the other heavy chain classes and subclasses (Maki et al., 1980; Kataoka et al., 1980, Sakano et al., 1980; Davis et al , 1980) In order to function after class switching, the immunoglobulin enhancer would have to be located upstream of the switch region, otherwise it would be deleted along with the C_μ coding sequence This is in fact the case since its location is more than 1 kb upstream of any known switch sites within the S_μ region. Thus as we have shown for the MOPC 141 γ_{2b} gene, it is likely that the same enhancer is used for the expression of all heavy chain classes following switch recombination, although it is possible that additional regulatory elements may be associated with the individual C region gene segments.

Evidence for Other Cellular Enhancer Elements

The data presented in this report represents the first clear demonstration of an enhancer element being associated with a defined cellular gene The possibility that enhancers are present in cellular DNA has already been suggested by others For example, Conrad and Botchan (1982) isolated human DNA sequences which hybridized to the region of SV40 DNA spanning the origin of replication One of these sequences was found to enhance the efficiency of tk transformation in an orientation-independent manner and thus resembles the viral enhancer elements Further more, this DNA sequence cross-hybridized with many sequences in human DNA, suggesting that a family of such elements exists

Rosenthal and Khoury (personal communication) have likewise isolated a human DNA sequence by virtue of its cross-hybridization with a portion of BK virus DNA. In this case, however, the sequence appears to be unique in the human genome even though it contains repeating 21 bp elements. These repeat elements show some homology to the BK virus enhancer region but appear to be about 8 times less active when tested for enhancing activity in the CAT assay of Gorman et al (1982).

Thus it is likely that enhancer elements might serve as a general mechanism for gene regulation in eucaryotes The association of such elements with specific genes is currently being studied in several systems. In addition to our demonstration of an enhancer element in the heavy chain immunoglobulin gene, two of us (V. O and S M) have found that sequences near the C_κ gene segment are essential for the high level expression of κ chains in transfected myeloma cells In this case, however. the functional similarity of this sequence element to the viral enhancers is less clear

The use of enhancer elements as regulators of gene

expression may not be confined to higher eucaryotic systems There is evidence (L Guarente, personal communication) that the yeast iso-1-cytochrome c gene is activated by heme and that sequences upstream of the gene are essential for this effect. Furthermore, the inversion of this activator sequence did not affect the inducibility of expression This strongly suggests that this region is not simply a component of the transcriptional promoter.

Tissue-specific Enhancer Elements and Their Possible Role in Cell Differentiation

The most interesting property of the immunoglobulin enhancer is its tissue specificity. The MSV and SV40 viral enhancers have been shown to have a certain degree of host cell specificity (Laimins et al. 1982) This effect may also be explained in terms of tissue specificity because the two cell types used for the comparison were derived from different tissues The immunoglobulin enhancer, on the other hand, functions at a high level in a lymphoid (myeloma) cell type but not at all in another cell type (fibroblast) of the same species. It is likely that this specificity is the result of a factor (or factors), present only in lymphoid cells, which regulates immunoglobulin expression during B cell ontogeny

At early stages of B cell development, μ heavy chains are expressed at a low level (Levitt and Cooper, 1980) After the B cell encounters antigen and interacts with regulatory T cells, terminally differentiated plasma cells appear and produce very high levels of immunoglobulin (Schibler et al, 1978) The quantitative differences in the level of immunoglobulin gene expression at different stages of B cell development suggest that the enhancer function may be stage-specific It is also possible that multiple regulatory elements are contained within this enhancer region and that increased levels of expression result from the combined effect of individual enhancers

Another possible example of a tissue-specific enhancer element has been described in the polyoma virus system (Katinka et al., 1980, Fujimura et al , 1981, Fujimura and Linney, 1982) It was shown that polyoma mutants that acquire the ability to replicate in the otherwise refractory F9 embryonal carcinoma cells contain point mutations and, in some cases, tandem duplications near the region of polyoma DNA which was shown (de Villiers et al , 1981) to have enhancer activity This result suggests that certain DNA sequences are recognized as an enhancer in cells permissive for polyoma virus but that sequences located at an adjacent site are recognized (after being mutated) in embryonal cells. Thus it appears that polyoma, like immunoglobulin genes, may contain multiple regulatory elements within their enhancer regions

The mechanism of tissue-specific enhancer is not known and this simply reflects our present ignorance of the mechanism of enhancers in general. Clearly though, the sequence differences and similarities between the various enhancers strongly suggest that specific regulatory proteins recognize these sites In fact, the glucocorticoid

receptor protein, which binds to the promoter region of mouse mammary tumor virus (MMTV), may be an example of an enhancer binding protein (K Yamamoto, personal communication) A sequence upstream of the MMTV promoter, essential for hormone responsiveness, was shown to enhance the herpes *tk* gene in an orientation–independent manner and to bind the hormone receptor

It is tempting to speculate that the presence or absence of such enhancer binding proteins determines whether or not an enhancer functions in a given cell type Furthermore, a particular enhancer binding protein might recognize many different but related sequences to a greater or lesser extent (or bind with different affinities) In this way it would be possible to activate many individual genes and to express them at different levels Alternatively, the level of expression of a gene that is controlled by an enhancer may be determined by the distance between this element and the promoter site. It seems likely that some or all of these mechanisms of gene regulation function during the process of cellular differentiation In this way the expression of multiple genes could be controlled (coordinately expressed) by a relatively small number of regulatory proteins

Enhancers as Activators of Cellular Oncogenes

One striking case for the role of enhancers in tumorigenesis was provided by studies of avian leukosis virus (ALV) induced chicken B cell lymphomas In such lymphomas, ALV DNA was found to be integrated adjacent to the *c-myc* gene (Payne et al , 1981), the cellular counterpart of the transforming sequences from the MC29 group of defective retroviruses (Sheiness and Bishop, 1979) Although it was first thought that *c-myc* was activated by a promoter-insertion mechanism (Hayward et al , 1981), it was later shown that ALV insertions could occur in the opposite orientation or downstream of the *c-myc* gene (Payne et al , 1982). Thus the ALV enhancer element was responsible for the increased level of *c-myc* expression and, presumably, for oncogenic transformation

The role of cellular enhancer elements in the activation of oncogenes has also been suggested by recent findings (reviewed by Klein, 1983) Many murine and human tumors of lymphoid origin have been shown to contain chromosomal translocations in which an oncogene (*c-myc*) has been rearranged to an immunoglobulin C region gene segment The majority of rearrangements in human Burkitt lymphomas were found to occur at the C_μ region while those in mouse plasmacytomas occur at the C_α region While the results we have described could account for the activation of *c-myc* in some human C_μ rearrangements (by analogy to the murine C_μ enhancer), the results in the murine system are somewhat unclear It has not been ruled out, however, that the murine C_α gene segment contains an additional enhancer element

We are currently investigating the activation of *c-myc* by sequences contained near the *c-myc*-C_μ junction in human DNA It will be interesting to compare the se-

quences in this region with those that we showed to contain enhancer activity Sequences that have been conserved through evolution may also help to identify the critical components of this regulatory element

was measured by labeling approximately 2×10^4 cells for 16 hr in 50 μl of normal growth medium containing ^{35}S-methionine (25 μCi/ml) Immunoglobulin was then immunoprecipitated from culture supernatants

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EXHIBIT H

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO
07/771,410	10/02/91	MORRISON	

RECEIVED

MAY 17 1993

FISH & NEAVE - PATENT DEPT.
REFERRED TO _VSV_
NOTED BY_____

VICKI S. VEENKER
C/O FISH & NEAVE
875 THIRD AVENUE
NEW YORK, NY 10022

	EXAMINER
	NISBET, T

ART UNIT	PAPER NUMBER
	15

DATE MAILED: 806

05/12/93

Below is a communication from the EXAMINER in charge of this application

COMMISSIONER OF PATENTS AND TRADEMARKS

ADVISORY ACTION

☒ THE PERIOD FOR RESPONSE:

a) ☒ is extended to run _6 months_ or continues to run _____ from the date of the final rejection

b) ☐ expires three months from the date of the final rejection or as of the mailing date of this Advisory Action, whichever is later. In no event however, will the statutory period for the response expire later than six months from the date of the final rejection

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee. The date on which the response, the petition, and the fee have been filed is the date of the response and also the date for the purposes of determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date of the originally set shortened statutory period for response or as set forth in b) above

☒ Appellant's Brief is due in accordance with 37 CFR 1.192(a)

☒ Applicant's response to the final rejection, filed _3/15/93_ has been considered with the following effect, but it is not deemed to place the application in condition for allowance:

1. ☐ The proposed amendments to the claim and/or specification will not be entered and the final rejection stands because

   a. ☐ There is no convincing showing under 37 CFR 1.116(b) why the proposed amendment is necessary and was not earlier presented

   b. ☐ They raise new issues that would require further consideration and/or search (See Note)

   c. ☐ They raise the issue of new matter. (See Note)

   d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal

   e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims

NOTE _____
_____
_____
_____

2. ☐ Newly proposed or amended claims _____ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☒ Upon the filing an appeal, the proposed amendment ☒ will be entered ☐ will not be entered and the status of the claims will be as follows:

   Claims allowed _____
   Claims objected to _____
   Claims rejected _____ 39 41 _____

   However,

   ☐ Applicant's response has overcome the following rejection(s) _____

4. ☒ The affidavit, exhibit or request for reconsideration has been considered but does not overcome the rejection because _the method of making does not structurally distinguish prior art antibodies from prior art antibodies, eg. monoclonal antibodies_

5. ☐ The affidavit or exhibit will not be considered because applicant has not shown good and sufficient reasons why it was not earlier presented

☐ The proposed drawing correction ☐ has ☐ has not been approved by the examiner.

☒ Other _Applicant's arguments may be persuasive with respect to method, but not for product because monoclonal antibodies are synthesized as a tetramers with proper glycosylation._

PTOL-303 (REV. 5-89)

Y. CHRISTINA CHAN
Y. CHRISTINA CHAN
PRIMARY EXAMINER
GROUP 1806

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER
	3

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Vicki Veenker

(2) Exmr Nisbet

(3) _____

(4) _____

Date of interview 12/10/93

Type: ☒ Telephonic   ☐ Personal (copy is given to   ☐ applicant   ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes   ☒ No.   If yes, brief description: _____

Agreement ☒ was reached with respect to some or all of the claims in question.   ☐ was not reached.

Claims discussed: N/A

Identification of prior art discussed: N/A

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Exmr's Amdt was agreed to

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview

☒ It is not necessary for applicant to provide a separate record of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

Examiner's Signature

PTOL-413 (REV 1-84)

Immunoglobulin gene expression in transformed lymphoid cells

(gpt/transformation)

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ABSTRACT   Myeloma, hybridoma, and thymoma cell lines have been successfully transfected for the Escherichia coli xanthine-guanine phosphoribosyltransferase gene (gpt) by using the plasmid vector pSV2-gpt. The transformed cells synthesize the bacterial enzyme 5-phospho-α-D-ribose-1-diphosphate:xanthine phosphoribosyltransferase (XGPRT; EC 2.4.2.22) and have been maintained in selective medium for over 4 months. Lymphoid cell lines expressing a κ immunoglobulin light chain were obtained by transfecting cells with pSV2-gpt containing a rearranged κ light chain genomic segment from the S107 myeloma cell line. The S107 light chain is synthesized in gpt-transformed J558L myeloma cells and is identical to the light chain synthesized by the S107 myeloma cell line, as judged by immunoprecipitation and two-dimensional gel electrophoresis. Furthermore, this light chain is synthesized and secreted as part of an intact antibody molecule by transformed hybridoma cells that normally secrete an IgG1 (γ,κ) antibody molecule. No light chain synthesis was detected in a similarly transformed rat myeloma or a mouse thymoma line.

Techniques to introduce novel genes into eukaryotic cells provide a powerful tool to study mechanisms of gene regulation and expression. Most studies on eukaryotic gene expression have been conducted in heterologous host cells—i.e., genes have been transfected into cell types (particularly human HeLa and mouse L cells) that normally do not express the gene of interest (1–3). Though a great deal has been learned about eukaryotic regulator sequences with these gene transfer experiments, it would be preferable to transfer genes encoding proteins expressed during differentiation back into the cell type that normally expresses the genes of interest The appropriate cell type provides protein modification systems, such as glycosyltransferases, necessary to make fully biological functional products. In addition, the appropriate cell type may be used to study tissue-specific regulation of gene expression.

To undertake studies of (i) the regulation and expression of immunoglobulin genes, (ii) the biosynthesis, chain-assembly, and secretion of immunoglobulin heavy and light chains, and (iii) structure–function correlates of antibody molecules, we have explored techniques for transfection of lymphoid cells using the pSV2-gpt vector (4, 5). This DNA can express the Eco gpt gene encoding xanthine-guanine phosphoribosyltransferase (XGPRT; 5-phospho-α-D-ribose-1-diphosphate:xanthine phosphoribosyltransferase, EC 2.4.2.22). Cells synthesizing XGPRT can be grown with xanthine as the sole precursor of guanine nucleotide formation (4, 5). Successfully transformed cells can be isolated by their ability to grow in medium containing xanthine and mycophenolic acid, an inhibitor of guanine nucleotide synthesis, if the transformed cell line is hypoxanthine phosphoribosyltransferase-negative (HPRT⁻; IMP pyrophosphate phosphoribosyltransferase, EC 2.4.2.8), transformants can be

selected in hypoxanthine/aminopterin/thymidine (HAT) medium (6). In the present experiments both calcium phosphate precipitation (7, 8) and protoplast fusion (9) techniques have been used to transfect cells.

pSV2-gpt containing a rearranged κ light chain gene (10) was used to transform several cultured lymphoid cell lines. Among the gpt transformants were clones that produce a new immunoglobulin light chain. The light chain produced by these transformed cell lines appears to be identical to the light chain synthesized by the myeloma cell from which the rearranged gene was isolated. Furthermore, in transformed hybridoma cells, this light chain is assembled with an immunoglobulin heavy chain and secreted as a complete antibody molecule.

MATERIALS AND METHODS

Cell Lines. J558L is a spontaneous heavy chain-loss-variant myeloma cell line obtained from the J558 cell line [α,λ, anti-α-1,3 dextran (11)] that synthesizes and secretes a λ light chain. V3-Ag1.2.3 is a HPRT⁻ rat myeloma cell line originally described by Galfre et al. (12) that synthesizes and secretes a rat κ light chain. 27-44 is a HPRT⁻ mouse IgG1 anti-dansyl hybridoma cell line (13), and BW5147 is a HPRT⁻, ouabain⁻ AKR thymoma originally described by Hyman and Stallings (14). Cell lines were maintained in either 10% newborn calf serum in Dulbecco's modified minimal essential medium (DME medium) or 10% fetal calf serum in alpha modified minimal essential medium.

Recombinant DNA Vectors. The plasmid vector pSV2-gpt has been described (4, 5). Fig 1 shows a partial restriction enzyme map of this vector. A second vector, which is derived from pSV2-gpt, but contains the herpes simplex thymidine kinase promoter inserted 5' of the gpt gene, was constructed by J -F Nicolas (unpublished data). pSV2-S107 was constructed by inserting a BamHI fragment containing the entire rearranged phosphocholine-specific κ chain gene from the S107 myeloma cell line (10) into the unique BamHI site in pSV2-gpt. The light chain gene is oriented so that the direction of transcription is opposite to the gpt gene (Fig. 1). The genomic rearranged S107 κ light chain DNA was a gift from M. Scharff.

Transfection by Protoplast Fusion. Protoplasts were prepared essentially as described by Sandri-Goldin et al. (9). Escherichia coli K-12 strain HB101, containing the appropriate plasmid, was grown at 37°C in Luria broth containing 1% glucose to an absorbance at 600 nm of 0.6–0.8. Chloramphenicol was added to 125 μg/ml, the culture was incubated at 37°C for 12–16 hr to amplify the plasmid copy number, and the cells were harvested by centrifugation. For every 25 ml of culture, 1 35 ml of chilled 20% sucrose/0.05 M Tris·HCl (pH 8) was added.

Abbreviations: XGPRT, xanthine-guanine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; HAT, hypoxanthine/aminopterin/thymidine; DME medium, Dulbecco's modified minimal essential medium.
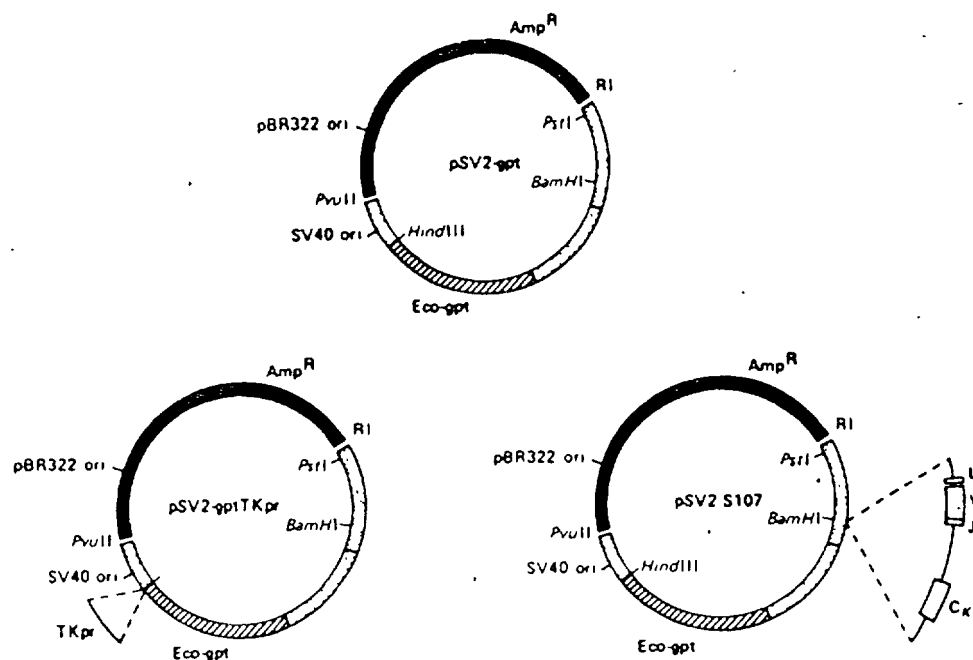
FIG. 1. Structure of the vectors used for lymphoid cell transformations. The diagram of the parental pSV2-gpt plasmid vector was taken from Mulligan and Berg (4, 5): pBR322 DNA is represented by the solid black lines and the plasmid's DNA replication origin and β-lactamase gene are indicated; the gpt gene sequence is represented by the hatched segments; simian virus 40 (SV40) sequences are the stippled segments. The SV40 origin of DNA replication (ori) and early promoter are located 5' of the gpt sequences. pSV2-gptTKpr has an insertion of 250 base pairs, containing the herpes simplex thymidine kinase promoter, between the gpt gene and the SV40 early promoter (unpublished data). pSV2-S107 has a 7-kilobase BamHI fragment, containing the entire genomic S107 light chain gene, inserted into the unique BamHI site of pSV2-gpt. This rearranged light chain gene is oriented in the opposite direction to gpt and contains the leader, V, and κ constant region exons as well as flanking 5' and 3' sequences.

the bacteria were suspended and 0.25 ml of lysozyme [a freshly prepared solution of 5 mg/ml in 0.25 M Tris·HCl (pH 8)] was added. After 5 min of incubation on ice, 0.5 ml of 0.25 M EDTA (pH 8) was added and incubation on ice was continued for an additional 5 min. After addition of 0.5 ml of 0.05 M Tris·HCl (pH 8), the bacteria were transferred to a 37°C water bath and were incubated for 10 min. At this time examination of the bacteria with a phase-contrast microscope showed that the vast majority had been converted to protoplasts. The bacteria were diluted with 10 ml of DME medium containing 10% sucrose and 10 mM MgCl$_2$ that was warmed to 37°C. After further incubation for 10 min at room temperature the protoplasts were ready for fusion.

Fusion of protoplasts with suspension cells was effected with a procedure normally used in the production of hybridomas (15). Cell lines were grown to a density of 0.3–1 × 10^6 cells per ml in DME medium supplemented with 10% newborn calf serum. Five milliliters of the protoplast suspension was added to 2 × 10^6 cells in growth medium. The mixture was centrifuged for 5 min at room temperature at approximately 500 × g. The supernate was aspirated and the pellet was resuspended gently in 2 ml of a polyethylene glycol solution [50 g of polyethylene glycol 1,500 (BDH) in 50 ml of DME medium] adjusted to pH 8 with CO$_2$. After 3 min of centrifugation at 500 × g the polyethylene glycol was diluted with 7 ml of DME medium while resuspending the pellet. After 5 min of centrifugation at 500 × g, the supernate was removed carefully and the cells were resuspended in DME medium containing 10% newborn calf serum and garamycin at 100 μg/ml and were plated either in 96-well or 24-well plates. After 48 hr, cells were diluted with an equal volume of DME medium containing xanthine at 250 μg/ml, hypoxanthine at 15 μg/ml, mycophenolic acid at 6 μg/ml, and 10% newborn calf serum. Every several days, as required, spent medium was aspirated carefully and was replaced with fresh medium containing the same supplements. Colonies of transformants were visible by 10 days. Transformants were maintained in selective medium.

Transfection by Calcium Phosphate Precipitation. Lymphoid cell lines grown in suspension were transfected by calcium phosphate precipitation as described by Chu and Sharp (7). Ten times concentrated HeBS buffer was stored at −20°C

until used, whereupon it was diluted to two times concentrated and adjusted to pH 7.05. Plasmid DNA (80 μg/ml) was made up in 125 mM CaCl$_2$ which was stored as a 2 M stock solution at −20°C. DNA-calcium phosphate precipitates were formed by dropwise addition of the DNA into the HeBS solution. The precipitate formed in 30 min at room temperature. The final DNA concentration was 40 μg/ml.

Cells were washed once in serum-free medium and were suspended directly in the DNA-calcium phosphate precipitate (10^6 cells per 20 μg of DNA per 0.5 ml). This suspension was incubated at 37°C for 30 min and then was diluted 1:10 in serum-containing medium. The cells were plated either into 24-well plates (2 × 10^5 cells per well) or 96-well plates (2 × 10^4 cells per well). Transfection of Y3 cells was done as described by Graham and Van der Eb (8) for adherent cell lines. The DNA-calcium phosphate precipitate was put directly onto the cell monolayer. After 30 min at 37°C, serum-containing medium was added. After 24 hr, half of the medium volume from each culture was removed and was replaced with fresh medium. On days 3, 4, 5, 8, 11, and 14, half the medium volume was removed and HAT medium was added. Transformed colonies were visible between 10 and 21 days.

Immunoprecipitations and Gel Electrophoresis. Immunoprecipitations were done with [^{35}S]methionine-labeled cell lysates and supernates. Biosynthetic labeling procedures have been described (16). Rabbit anti-mouse light chains, rabbit anti-mouse κ light chains, rabbit anti-mouse immunoglobulin, and a hybridoma anti-mouse IgGf allotype antibody were used for immunoprecipitations. Staphylococcus aureus, Cowan strain 1 (IgGsorb, Enzyme Center, Boston) was used to coprecipitate the antigen–antibody complexes (16).

One-dimensional NaDodSO$_4$/polyacrylamide slab electrophoresis and two-dimensional nonequilibrium gradient gel electrophoresis were done as described (17). Autoradiography of polyacrylamide gels was with preflashed XAR-5 film and fluorography by using sodium salicylate (18).

RESULTS

Transfection Frequencies. The frequency at which stable transformed lymphoid cell lines were generated was influenced

by every parameter tested. Different cell lines and different vectors produced different transformation frequencies. Moreover, the two DNA delivery procedures, protoplast fusion and calcium phosphate precipitation, yielded different transformation frequencies. Tables 1 and 2 summarize the results by using protoplast fusion and calcium phosphate precipitation, respectively.

Under the present experimental conditions, BW5147 appears to be the least competent recipient of the cell lines tested, having a transformation frequency of approximately 10^{-6}. Y3 and 27-44 yielded frequencies in the range of 0.3 to >5 × 10^{-6}. In the present experiments, J558L yielded the highest frequency with the range of 3 × 10^{-6} to >10^{-4}. Protoplast fusion appears on balance to be a more efficient delivery system than calcium phosphate precipitation.

A striking feature of these results is the enhanced transformation frequency for gpt obtained with the light chain-containing vector, pSV2-S107. This dramatic increase is evident when the pSV2-S107 vector was used with the J558L and Y3 myeloma cell lines; transformation with this recombinant was 5- to at least 10-fold greater than that obtained with the other vectors. Transformation of the hybridoma 27-44 cell line was increased only about 2-fold with pSV2-S107. The sequence(s) in the pSV2-S107 insert that is responsible for the enhanced transformation frequency must yet be mapped. Transformation of the Y3 cell line was occasionally greater with pSV2-gptTKpr than with pSV2-gpt (Table 2). Regardless of which vector was used, BW5147 transformants were detected only at very low frequencies. The amount of XGPRT activity in cell lines stably transformed by the three recombinant plasmids was not significantly different (Fig. 2 and data not shown).

XGPRT Activity. The transformed cell lines expressed the Eco gpt gene, as measured by the presence of XGPRT activity in the cell lysates. E. coli XGPRT can be distinguished from mammalian HPRT activity by its different electrophoretic mobility (4, 5). In cells selected for resistance to mycophenolic acid, both the cellular HPRT and bacterial XGPRT activities were detectable (Fig. 2). Cells lacking their own HPRT activity and selected for gpt in HAT medium had only the bacterial enzyme activity (Fig. 2).

Immunoglobulin Light Chain Expression. The organization of exons in the S107 genomic light chain gene is shown in Fig 1. To produce the S107 light chain protein from this gene, two introns must be processed from the primary mRNA transcripts and the leader polypeptide removed by post-translational cleavage. For secretion of the light chain as part of an intact antibody molecule, the newly synthesized light chain must fold and assemble with an immunoglobulin heavy chain to form an H_2L_2 tetramer. This also involves the formation of interchain disulfide bonds.

Table 1. Transformation of lymphoid cell lines with the pSV2-gpt vectors by using protoplast fusion

| Vector | Cell line | |
	J558L	BW5147
pSV2-gpt	21/288* 27/36†	0/76* 1/48†
pSV2-gptTKpr	10/190* 24/36†	0/80* 1/48†
pSV2-S107	186/192* 36/36†	4/96* 8/48†
	149/192‡	

Results are from three experiments and are expressed as the number of culture wells having stable transformants.
* After protoplast fusion cells were plated in 96-well culture dishes at 10^4 cells per well.
† Cells were plated at 10^5 cells per 2.0 ml of culture in 24-well dishes.
‡ Cells were plated at 5 × 10^3 cells per well in 96-well culture dishes.

Table 2. Transformation of lymphoid cell lines with the pSV2-gpt vectors by using calcium phosphate precipitation

| Vector | Cell line | | |
	Y3	27-44	BW5147
pSV2-gpt	3/48*	10/192†	1/192†
pSV2-gptTKpr	19/48*	10/192†	1/192†
pSV2-S107	47/48*	43/288†	0/192†

Results are from three experiments with the Y3 cell line and two experiments with 27-44 and BW5147 cell lines.
* Cells were plated in 24-well culture dishes at 2 × 10^5 cells per well.
† Cells were plated in 96-well culture dishes at 4 × 10^4 cells per well.

Of the four cell lines stably transformed with pSV2-S107, the J558L cell line synthesized, but did not secrete, the S107 κ light chain. However, this cell line was not expected to secrete the newly made light chains because heavy chain-loss-variants of the S107 myeloma cell line also do not secrete endogenous light chains (M. Scharff, personal communication) Transformants of the 27-44 hybridoma cell line synthesized and secreted the S107 light chain. Moreover, the S107 light chain was assembled into tetrameric H_2L_2 immunoglobulin molecules with the endogenous γl-heavy chain and was secreted Twelve independently transformed Y3 and seven BW5147 cell lines did not produce detectable amounts of the S107 light chain, as judged by immunoprecipitation and gel analyses. XGPRT analyses verified that these cells were, indeed, transformants.

Autoradiograms of two-dimensional polyacrylamide gels showing the apparent M_r and charge of the light chains produced by J558L and 27-44 transformants are shown in Figs. 3 and 4. The two-dimensional gel pattern of the S107 light chain synthesized by S107 myeloma cells is included to show that the transformed cell lines produced a light chain that is identical in apparent M_r and charge. The two-dimensional gel patterns also show that the leader polypeptide was removed in transformed cell lines that expressed the light chain. This indicates that proper transcription, mRNA, and protein processing occur in the transformants. Transcription of the S107 light chain gene probably occurs from its own promoter, because the light chain gene is oriented opposite to the direction of the SV40 early promoter (see Fig. 1).

The antibodies secreted by 27-44 transformants were immunoprecipitated with both hybridoma anti-IgC1 allotypic antibody and rabbit anti-mouse light chain antisera Both reagents precipitated the S107 light chain (data not shown). Sequential precipitation, first with the hybridoma anti-IgC1 antibody and
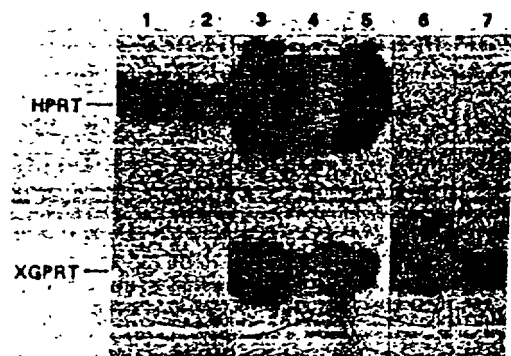

FIG. 2. XGPRT and HPRT production in transformed lymphoid cell lines. Enzyme analyses were done as described by Mulligan and Berg (4, 5). Lanes: 1 and 2, electrophoretic mobility of mammalian HPRT; 3–5, J558L cell transformants; and 6 and 7, transformants of the 27-44 cell line. Because 27-44 is a HPRT⁻ cell line, only XGPRT is present.

$M_r \times 10^{-3}$ BASIC   ACIDIC
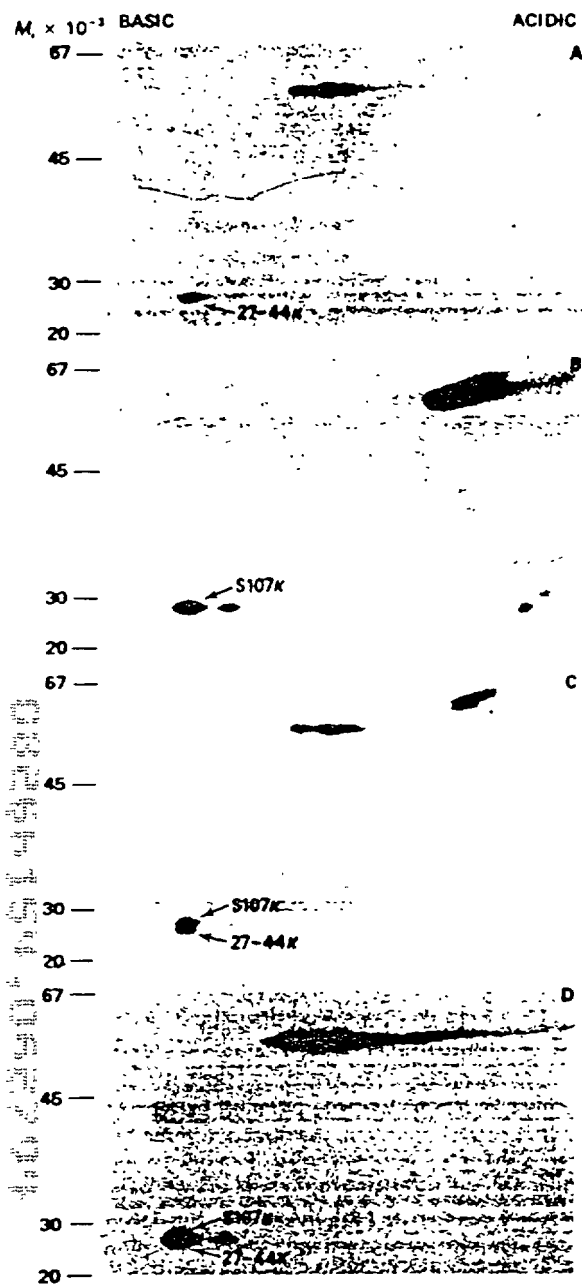
FIG. 3. S107 light chain produced by transformed 27-44 cells. Autoradiograms of two-dimensional gels of the light and heavy chains produced by parental and transformed cell lines are shown. (A) Parental 27-44 IgG1 anti-dansyl antibody immunoprecipitated with an anti-IgG1-specific hybridoma antibody. Both the γ1 heavy and κ light chains can be seen. (B) S107 IgA antibody immunoprecipitated with a rabbit anti-IgA antiserum. The α heavy chain was distinguished clearly by charge and apparent M_r from the γ1 heavy chain in A. (C) A mixture of the immunoprecipitates of A and B. The two κ light chains can be seen as distinct spots (indicated by arrows) having nearly identical charge but different apparent M_r. (D) Immunoprecipitate of a transformed 27-44 cell line. Only the γ1 heavy chain was present, but two light chains can be seen. In this case the amount of S107 light chain was considerably lower than in the artificial mixture shown in C.

then with the rabbit anti-κ antisera, indicated that little, if any, free S107 light chain was secreted by these cells This shows that the S107 light chain is assembled with the γ1 heavy chain into an intact antibody molecule.

Different amounts of S107 light chains were produced when a number of independent J558L and 27-44 transformants were
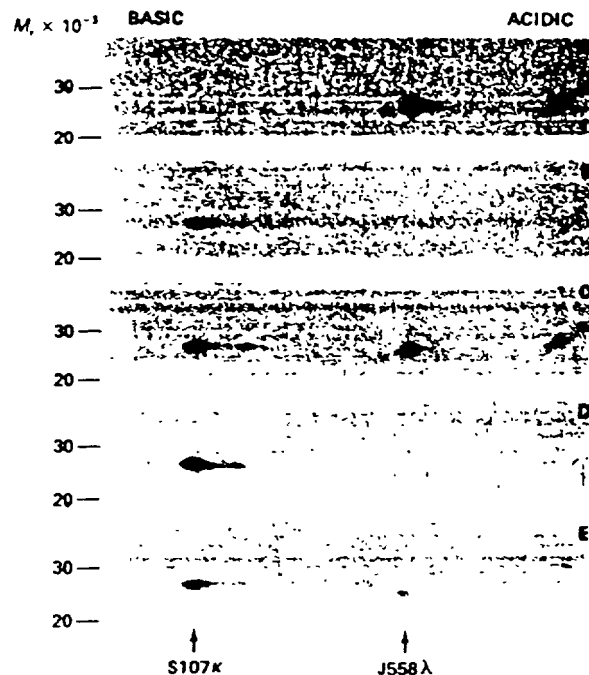
FIG. 4. S107 light chain produced by transformed J558L cells. Autoradiograms of two-dimensional gels of light chains immunoprecipitated from cell lysates of J558L transformed with pSV2-S107 DNA are shown Because J558L does not produce a heavy chain, only the light chain portions of the two-dimensional gels are shown. (A) λ light chain produced by the parental J558L cell line. (B) S107 κ light chain. (C) A mixture of the two light chains. The κ and λ light chains are distinguished on the basis of both charge and apparent M_r. (D and E) Two independently derived J558L cell lines transformed with pSV2-S107 DNA The transformant examined in D only appears to produce larger quantities of the S107 κ light chain than the endogenous J558 λ light chain, because the S107 κ chain is synthesized and remains in the cytoplasm, while the J558 λ chain is synthesized and secreted.

compared. Amounts varied from barely detectable to quantities equal to endogenous light chain. This variation may be due to the chromosomal region where the light chain has integrated. It also could result from different copy number of the light chain gene in different transformants. Quite possibly, mutations or deletions of sequences needed for the expression of this gene could have occurred during transformation or subsequent to integration of the light chain sequence. Further studies are needed to determine the cause of this variation and why light chain expression does not occur in Y3 or BW5147 cell lines transformed with the same light chain gene vector.

DISCUSSION

These experiments show that it is possible to use two methods, calcium phosphate precipitation and protoplast fusion, to introduce genes into lymphoid cells. With pSV2-gpt containing the gene for an immunoglobulin light chain (pSV2-S107) both methods give rise to transformants that synthesize bacterial XGPRT and the murine light chain. Higher transformation frequencies are seen following protoplast fusion. Indeed, by using protoplast fusion and the pSV2-S107 plasmid, transformants can be obtained at a frequency of greater than 10^{-4}. Transformation frequencies are lower when using the other plasmids or calcium phosphate precipitation. Because mycophenolic acid resistance or reversion of the HPRT⁻ phenotype do not occur spontaneously in the cell lines used, stable transformation, at even low frequencies, can be detected.

A surprising result is the increased frequency of gpt transformation when the S107 light chain is incorporated into the

pSV2-gpt vector. This enhancing effect occurs with both the rat and mouse myelomas. A similar increased transformation frequency has been observed with a bovine papillomavirus vector containing the human β-globin region sequences (19). At present, the mechanism for the increased transformation frequency in both cases is obscure. Possibly, the chromosomal DNA provides an origin of DNA replication, which permits the plasmid to replicate within the transformed cell and increases the transformation frequency. Transcription from the immunoglobulin promoter cannot be essential for the increased transformation frequencies because deletion of the fragments that are presumed to contain the immunoglobulin promoter region does not abolish the enhancement of transformation. It also is possible that pSV2-S107 is more efficient for transformation because of increased XGPRT production; this seems unlikely because there are no consistent differences in enzyme levels in the stable transformants obtained with either vector.

DNA-mediated gene transfer into lymphoid cells may permit a study of the regulation and expression of immunoglobulin genes in cells in which they normally are synthesized. It may be possible to examine the basis for differential immunoglobulin gene expression at different stages of lymphocyte differentiation. Cell lines in which immunoglobulin synthesis ca n be induced (20–22) are suitable hosts to determine if the transduced immunoglobulin genes also are responsive to those signals. Studies with cells transformed with genetic elements that are inducible by steroid hormones demonstrate that transduced DNA can respond, if the cell contains the appropriate receptors (23).

A question of central importance is what determines the utilization of various promoters and thus the synthesis of defined proteins in certain cell lines. In our experiments light chains are efficiently produced in both transformed mouse myeloma and hybridoma cell lines. However, light chain production did not occur in either a rat myeloma or a mouse thymoma. The inability of the immunoglobulin promoter to function in a different species has been reported by Falkner and Zachau (24). The lack of production of mouse immunoglobulin in a rat myeloma is surprising because mouse myelomas have been used to fuse to rat myelomas to produce hybrid cells that synthesize both rat and mouse immunoglobulin molecules (25). The possibility that the S107 light chain is synthesized but rapidly degraded in the Y3 myeloma has not been excluded.

There is evidence that differentiated cell types express immunoglobulin genes to varying levels. For example, somatic cell hybridization of myelomas yields hybridomas that produce antibodies, whereas thymomas yield hybrid cells with T-cell phenotypes (26). Furthermore, hybridization of myelomas with non-B cells results in cessation of immunoglobulin production (26, 27). The lack of light chain expression in the transformed thymoma may reflect tissue-specific gene regulation. It is important to determine if immunoglobulin gene expression in the nonexpressing mouse thymoma and rat myeloma cell lines is regulated at the level of transcription, RNA processing, translation, or rapid protein turnover.

The study of the structure and function of the immunoglobulin molecule has been of great interest, both because of the ability of immunoglobulin to react with a diverse family of ligands and also because of the biologic importance of antibody molecules. Initially, the study of immunoglobulins was limited to the study of heterogeneous serum pools after immunization. The advent of myelomas, and more recently hybridomas, has permitted the study of homogeneous populations of antibodies. DNA-mediated transfection and immunoglobulin gene expression is an important tool to permit the study of immunoglobulin

molecules. By using this technique, it should be possible to study the function of both novel chain combinations and novel chain structures. In vitro site-specific mutagenesis techniques can be used to construct specific mutations in immunoglobulin genes that can be expressed after transfection. Because significant quantities of immunoglobulin are produced in the transformants, sufficient quantities of protein necessary for detailed analyses should be obtained.

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Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*

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IN antibodies, a heavy and a light chain variable domain, VH and VL, respectively, pack together and the hypervariable loops on each domain contribute to binding antigen[1-4]. We find, however, that isolated VH domains with good antigen-binding affinities can also be prepared. Using the polymerase chain reaction[5], diverse libraries of VH genes were cloned from the spleen genomic DNA of mice immunized with either lysozyme or keyhole-limpet haemocyanin. From these libraries, VH domains were expressed and secreted from *Escherichia coli*. Binding activities were detected against both antigens, and two VH domains were characterized with affinities for lysozyme in the 20 nM range. Isolated variable domains may offer an alternative to monoclonal antibodies and serve as the key to building high-affinity human antibodies. We suggest the name 'single domain antibodies (dAbs)' for these antigen binding demands.

We have analysed the interactions with antigen of individual domains of the anti-lysozyme antibody, D1.3 (ref. 1). The VH domain was expressed in *E. coli* and secreted into the periplasm[6,7], alone or in association with the Vκ domain (fig. 1). Analysis of culture medium, by passage through a lysozyme-Sepharose affinity column[8], followed by SDS-PAGE[9] revealed that both the isolated VH domain, or the associated Fv fragment, could bind to lysozyme, and could be purified to homogeneity in a single step, with yields of ~200 μg l⁻¹ and 10 mg l⁻¹, respectively. The VH domain appears to be monomeric by FPLC (Pharmacia, Superose 12 column). The N-terminal sequences of both domains were checked by gas-phase protein sequencing[10,11].

As shown in Table 1, the affinity of Fv fragment for lysozyme and the stoichiometry of binding of the VH domain to lysozyme were determined by titration using fluorescence quench techniques. The affinity of VH domain for lysozyme was determined

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TABLE 1   Affinities of Fv fragment and VH domains for hen egg lysozyme

	Stoichiometry	Affinity (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	k_{off}/k_{on} (nM)
Fv-D1 3	ND	3	1.9×10^6	ND	ND
VH-D1 3	1 2	ND	3.8×10^6	0.075	19
VH3	ND	ND	2.9×10^6	0.036	12
VH8	ND	ND	3.3×10^6	0.088	27

Cultures of 500 ml were grown and induced (see Fig 1 methods), and the supernatant passed through a 0.45 μm filter (Nalgene), then through a 5 ml lysozyme-Sepharose affinity column After washing with phosphate buffered saline (PBS), the Fv fragment or VH domains were eluted with 50 mM diethylamine, and analysed for purity by SDS-PAGE[9]. The proteins were titrated with lysozyme at 25 °C using fluorescence quench (Perkin Elmer LS 5B Luminescence Spectrometer)[27] to determine the number of active binding sites, to measure the affinity of the Fv fragment and the stoichiometry of binding of the VH domain (mole lysozyme per mole domain) The concentration of the VH domain of the D1 3 antibody was determined by hydrolysis followed by quantitative amino-acid analysis The kinetics of lysozyme binding were determined by stopped-flow (HI Tech Stop Flow SHU) at 20 °C under pseudo-first order conditions with binding sites in five to ten fold excess over lysozyme[28]. For the kinetics, the concentration of binding sites, not protein, was measured ND, not determined. k_{on} is the second order rate constant for association, and k_{off} is the first order rate constant for dissociation

from the kinetics of binding. The affinity of the Fv fragment (3 nM) is similar to the parent antibody (2 nM). The VH domain binds lysozyme tightly in an equimolar complex with an affinity for lysozyme (19 nM) which is only 10-fold weaker. Separated heavy and light chains have previously been identified with antigen[12] or hapten binding activities[13] although the affinities were poor, with no evidence for binding by single chains[13,14] rather than dimers[15].

In the D1.3 antibody, lysozyme interacts extensively with both domains, and forms three hydrogen bonds to the Vκ domain, and nine hydrogen bonds to the VH domain. Binding of lysozyme buries ~300 Å² of Vκ domain away from solvent, and 400 Å² of the VH domain[1]. Our results show, however, that the Vκ domain makes only a small net contribution to the energetics of binding. This is surprising as the removal of a single hydrogen bond[16] or a single van der Waals contact[17] can lead to tenfold loss in affinity. The VH domain presumably binds to lysozyme in a similar way to the antibody, and this is consistent with inhibition of binding of the Fv fragment by the VH domain (data not shown, but see Fig. 1 legend). It is possible that the whole surface of interaction might reorientate slightly, perhaps by rocking on side chains, to create a new set of contacts[18].

The result prompted us to obtain VH domains with antigen-binding activities from antibody-producing cells. Previously we have demonstrated the cloning of immunoglobulin variable regions from hybridoma mRNA for expression of chimaeric antibodies, using the polymerase chain reaction (PCR)[5,19]. We now used PCR to amplify the rearranged VH genes from the spleen DNA of a mouse immunized with lysozyme (Fig. 2). The amplified DNA was cloned into the vector M13VHPCR1 (ref. 19) for sequencing. The complete sequences of 48 VH gene clones were determined (data not shown). All but two of the mouse VH gene families[20] were represented, with frequencies of: VA (1), IIIC (1), IIIB (8), IIIA (3), IIB (17), IIA (2), IB (12) and IA (4). In 30 clones the D segments could be assigned to families SP2 (14), FL16 (11) and Q52 (5), and in 38 clones the JH minigenes to families JH1 (3), JH2 (7), JH3 (14) and JH4 (14). The different sequences of CDR3 marked each of the 48 clones as unique. Nine pseudogenes and 16 unproductive rearrangements were identified; of the clones sequenced, 27 have open reading frames. Clearly we can generate a diverse repertoire of VH genes using PCR, but cannot rule out a systematic bias due to our choice of primers and hybridization conditions. VH gene libraries have also been generated using PCR

FIG. 1 Vectors for expression of VH and Vκ domains Scheme showing inserts in expression vectors. *a,* pSW1-VHD1 3: *b,* pSW1-VHD1 3-VKD1 3, *c,* pSW1-VHD1.3-TAG1; *d,* pSW1-VHD1.3-VKD1 3-TAG1; *e,* pSW1-VHPOLY, *f,* pSW1-VHPOLY-TAG1; and *g,* nucleotide sequence of pSW1-VHPOLY-TAG1 insert. The amino acid sequence of the pelB leader and TAG1 are shown in italics

METHODS. The vectors were assembled from pUC19 (ref. 29), synthetic oligonucleotides encoding the pelB signal sequence[30] peptide tag[24], restriction site polylinker, and (as appropriate) cloned cDNA of the VH and Vκ domains of the D1.3 antibody (M. E. Verhoeyen, C. Berek and G. W., unpublished data) Recombinant plasmids were transformed into *E. coli* BMH71-18 (ref 31). colonies selected on TYE plates[32] with 100 μg ml⁻¹ ampicillin (AMP) and 1% glucose (GLU), and toothpicked into 200 μl 2 × TY medium[32], AMP, GLU in wells of ELISA plates Colonies were grown at 37 °C for 16-24 h Cells were pelleted, washed twice in 50 mM NaCl (200 μl) and resuspended in 200 μl 2 × TY medium, AMP and 1 mM isopropylthiogalactoside (to induce expression) and grown for a further 16-24 h The cells were cooled, pelleted and supernatants screened for secretion of VH domains (by western blotting) or antigen binding activity (by direct ELISA). Western blot methods were essentially as in ref 22· supernatant (10 μl) from the cultures was subjected to SDS-PAGE[9] and proteins then transferred electrophoretically to nitrocellulose The VH domains were detected by means of the peptide tag with 9E10 antibody[24] However, the tag can be lost by proteolytic cleavage in culture, especially after prolonged growth of the bacteria Bound antibody was detected using horseradish peroxidase conjugated rabbit anti-mouse antibody at a dilution of 1 1000 4-chloro-1-naphthol (Sigma) was used as the peroxidase substrate. For direct ELISA, wells of Falcon ELISA plates were coated with antigen in phosphate buffered saline (PBS) overnight (3 mg ml⁻¹ lysozyme, or 50 μg ml⁻¹ KLH), then blocked with 2% skimmed milk powder in PBS for 2 h at 37 °C. Bacterial supernatant was added and incubated at 37 °C for 2 h. D1.3-VH domains were detected with rabbit polyclonal antiserum raised against the D1.3 Fv fragment, using peroxidase conjugated goat anti-rabbit immunoglobulin Tagged VH domains were detected as described in western blotting except with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) as the peroxidase substrate Three washes of 0.05% Tween 20 in PBS, were followed by three washes of PBS between each step (only PBS washes before addition of blocker or bacterial supernatants). Competition ELISA was as above except that the binding of the Fv fragment tagged on the Vκ domain was competed against by untagged VH domain.

from mRNA of human peripheral blood lymphocytes (J. Marks, D.G. & G.W., unpublished data) and from mRNA of mouse spleen[21].

Amplified DNA was then cloned for expression into a vector which incorporates a C-terminal peptide tag to facilitate detection of expressed VH domains (Fig. 1*f*). Bacterial supernatants were analysed by SDS-PAGE followed by western blotting[22], and bands of the expected size ($M_r \approx 14,000$) were detected for 14 of the 17 clones by probing with antibody directed against the tag[23,24]. To screen for lysozyme binding activities, about two thousand colonies were toothpicked in groups of five into wells of enzyme-linked immunosorbent assay (ELISA) plates, and the supernatants tested for binding to lysozyme-coated plates. Twenty-one supernatants were shown to have lysozyme-binding activity, and some of the corresponding individual clones were prepared.

Two of the clones (VH3 and VH8) with lysozyme-binding activities were sequenced (Fig. 3). They belonged to the same VH-gene (Kabat IIB) families and D-segment families (FL16) but had different J segments (JH2 and JH4). There were only six amino-acid differences between the (unrearranged) VH genes, but the sequences of CDR3 were completely different. The VH domains were purified and affinities for lysozyme determined (Table 1). The affinities, in the 20 nM range, are similar to those of the VH domain of the D1.3 antibody. To check the specificity of binding, the three VH domains were also screened for binding to four other purified proteins (bovine serum albumin, insulin, keyhole-limpet haemocyanin (KLH) and cytochrome c), and to foetal calf serum, milk powder and plastic of microtitre plates. No binding was detected. (However, other cross-reactive VH domains have been found.) The VH-D1.3, VH3 and VH8 domains appear to bind to the same region of lysozyme, as they inhibit the binding of the D1.3 Fv fragment.
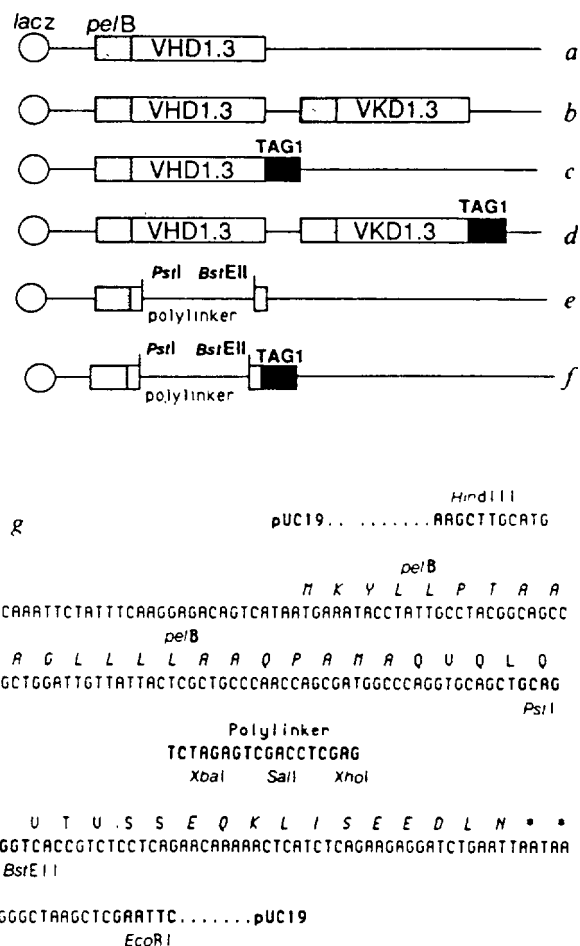
To test whether VH domains with other binding activities

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pUC19.........AAGCTTGCATG *HindIII*

pelB
M K Y L L P T A A
CAAATTCTATTTCAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
pelB
A G L L L L A A Q P A M A Q U Q L Q
GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAG
PstI

Polylinker
TCTAGAGTCGACCTCGAG
XbaI   *SalI*   *XhoI*

U T U S S E Q K L I S E E D L N * *
GGTCACCGTCTCCTCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATTAATAA
BstEII

GGGCTAAGCTCGAATTC.......pUC19
EcoRI

FIG 2 PCR amplification of VH genes from mouse genomic DNA Agarose gel electrophoresis of amplified mouse VH DNA (left-hand lane) with markers OX174 *Hae*III fragments (right-hand lane). METHODS Balb/c mice were hyperimmunized with hen egg-white lysozyme (100 μg antigen, intraperitonally on day 1 in complete Freunds adjuvant and on day 14 in incomplete Freunds adjuvant, followed by 50 μg antigen intravenously on day 35, mice were killed on day 39) or similarly with KLH. DNA was prepared from the spleen and the rearranged mouse VH genes were amplified. Conditions were chosen to minimize annealing between the 3' ends of the two primers. The sample (50-100 μl) included 50-200 ng DNA, VH1FOR-2 (5' TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3') and VH1BACK primers[19] (25 pm of each) 250 μM of each dNTP, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 100 μg ml⁻¹ gelatine The sample was overlaid with paraffin oil, heated to 95 °C for 2 min, 65 °C for 2 min, and then to 72 °C; *Taq* polymerase (2 units, Cetus) was added after the sample had reached the elongation temperature and the reaction continued for 2 min at 72 °C The sample was subjected to a further 29 rounds of temperature cycling using the Techne PHC-1 programmable heating block The amplified DNA was digested with *Pst*I and *Bst*EII and fractionated on an agarose gel A band of about 350 base pairs was extracted and cloned

could be made, and whether immunization was necessary, a new VH-gene library was prepared from a mouse immunized with KLH. Culture supernatants from the two libraries were tested for binding to lysozyme or to KLH. The first library (immunization with lysozyme) had yielded 21 supernatants with lysozyme- and two with KLH-binding activities, whereas the second library (immunization with KLH, screening ~2,000
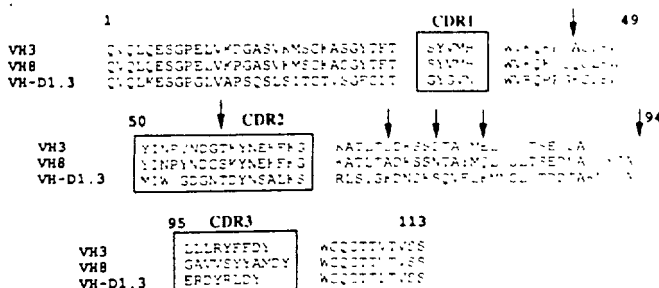
FIG. 3 Sequences of VH domains with lysozyme-binding activities. The sequences of the VH domains are aligned with that of the D1 3 antibody

colonies) yielded two supernatants with lysozyme- and 14 with KLH-binding activities. We conclude that VH domains can be derived, preferably after immunization, with binding activities to lysozyme and KLH and presumably other antigens. VH domains lack the cavity which can be formed with the V_κ partner however, and this might bias the binding activities against hapten binding[2,15]. The affinity of the VH domains (20 nM or $5 \times 10^7 M^{-1}$) for lysozyme lies within the range expected for the affinities of monoclonal antibodies for protein antigens, and can be improved by site-directed mutagenesis (unpublished data).

VH domains with binding activities can be generated in a matter of days without recourse to tissue culture, and may also have other advantages over monoclonal antibodies. For example, the smaller molecule should penetrate tissues more readily, could permit the blocking of 'canyon' sites on viruses[25,26] and allow epitope mapping at higher resolution. However, VH domains are relatively 'sticky', presumably due to the exposed hydrophobic surface normally capped by the V_κ or $V\lambda$ domains. It should be possible to design VH domains with improved properties. We also envisage that VH domains with binding activities could serve as the building blocks for making Fv fragments or complete antibodies. For example, such VH domains could be co-expressed with a repertoire of V_κ domains, derived by PCR amplification of V_κ genes[19] and screened for association of the domains and antigen binding. This approach could prove valuable for building human antibodies of therapeutic value, and even for catalytic antibodies; for example, making Fv fragments in which the VH domain binds substrate, and side chains or prosthetic groups in the V_κ partner stabilize the transition state or attack the substrate[33]. □

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Protease nexin-II, a potent anti-chymotrypsin, shows identity to amyloid β-protein precursor

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PROTEASE nexin-II (PN-II) is a protease inhibitor that forms SDS-resistant inhibitory complexes with the epidermal growth factor (EGF)-binding protein, the γ-subunit of nerve growth factor, and trypsin[1-3]. The properties of PN-II indicate that it has a role in the regulation of certain proteases in the extracellular environment. Here we describe more of the amino-acid sequence of PN-II and its identity to the deduced sequence of the amyloid β-protein precursor (APP)[4,5]. Amyloid β-protein is present in neuritic plaques and cerebrovascular deposits in individuals with Alzheimer's disease and Down's syndrome[6-9]. A monoclonal antibody against PN-II (designated mAbP2-1) recognized PN-II in immunoblots of serum-free culture medium from human glioblastoma cells and neuroblastoma cells, as well as in homogenates of normal and Alzheimer's disease brains. In addition, mAbP2-1 stained neuritic plaques in Alzheimer's disease brain. PN-II was a potent inhibitor of chymotrypsin with an inhibition constant K_i of 6×10^{-10} M. Together, these data demonstrate that PN-II and APP are probably the same protein. The regulation of extracellular proteolysis by PN-II and the deposition of at least parts of the molecule in senile plaques is consistent with previous reports that implicate altered proteolysis in the pathogenesis of Alzheimer's disease[4,5,10-12].

Protease nexins are protein protease inhibitors that are synthesized and secreted by various cultured extravascular cells. They form SDS-resistant complexes with certain serine proteases; the complexes then bind back to the cells and are rapidly internalized and degraded[1-3,13,14]. Protease nexin-I is a specific thrombin inhibitor when bound to the cell surface[15]. It stimulates neurite outgrowth in cultured neuroblastoma cells[16,17] and primary chick sympathetic neurons[18] and is reduced by about six-fold in Alzheimer's disease brain[19]. PN-II is unusually stable and forms complexes with target proteases even after incubation of the protein with SDS or at a pH of 1.5 (ref. 3).

We analysed the N-terminal amino-acid sequence of two peptides obtained from digestion of PN-II. Sequencing of a CNBr peptide of PN-II yielded a sequence that overlapped with the N-terminal sequence previously reported[3] (Fig. 1). Additional sequence was obtained from a PN-II peptide generated by endoproteinase Lys-C. We searched the protein-sequence database of the National Biomedical Research Foundation for sequences with identity to the known sequence of PN-II. Identity was found with the deduced sequence for APP[4,5] (Fig. 1). The only discrepancy was amino-acid residue 27 in PN-II, which we originally reported as a questionable phenylalanine[3]; there

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